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THE CHEMICAL ASPECTS OF IMMUNITY

BY

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SECOND (REVISED AND ENLARGED) EDITION

American Chemical Society
Monograph Series

BOOK DEPARTMENT
The CHEMICAL CATALOG COMPANY, *Inc.*
419 FOURTH AVENUE, AT 29TH STREET, NEW YORK, U. S. A.
1929

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GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in coöperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company of New York City.

The Council, acting through the Committee on National Policy of the American Chemical Society, appointed the editors, named at the close of this introduction, to have charge of securing authors, and of considering critically the manuscripts prepared. The editors of each series will endeavor to select topics which are of current interest and authors who are recognized as authorities in their respective fields. The list of monographs thus far secured appears in the publisher's own announcement elsewhere in this volume.

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The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years and the fields covered by this development have been so varied that it is difficult for any individual to keep in touch with the progress in branches of science outside his own specialty. In spite of the facilities for the examination of the literature given by Chemical Abstracts and such compendia as Beilstein's Handbuch der Organischen Chemie, Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Chemie, Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Chemie and the English and French Dictionaries of Chemistry, it often takes a great deal of time to coördinate the knowledge available upon a single topic. Consequently when men who have spent years in the study of important subjects are willing to coördinate their knowledge and present it in concise, readable form, they perform a service of the highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviews of this character that a Committee of the American Chemical Society recommended the publication of the two series of monographs under the auspices of the Society.

Two rather distinct purposes are to be served by these monographs. The first purpose, whose fulfilment will probably render to chemists in general the most important service, is to present the knowledge available upon the chosen topic in a readable form, intelligible to those whose activities may be along a wholly different line. Many chemists fail to realize how closely their investigations may be connected with other work which on the surface appears far afield from their own. These monographs will enable such men to form closer contact with the work of chemists in other lines of research. The second purpose is to promote research in the branch of science covered by the monograph, by furnishing a well digested survey of the progress already made in that field and by pointing out directions in which investigation needs to be extended. To facilitate the attainment of this purpose, it is intended to include extended references to the literature, which will enable anyone interested to follow up the subject in more detail. If the literature is so voluminous that a complete bibliography is impracticable, a critical selection will be made of those papers which are most important.

The publication of these books marks a distinct departure in the policy of the American Chemical Society inasmuch as it is a serious attempt to found an American chemical literature without primary regard to commercial considerations. The success of the venture will depend in large part upon the measure of coöperation which can be secured in the preparation of books dealing adequately with topics of general interest; it is earnestly hoped, therefore, that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

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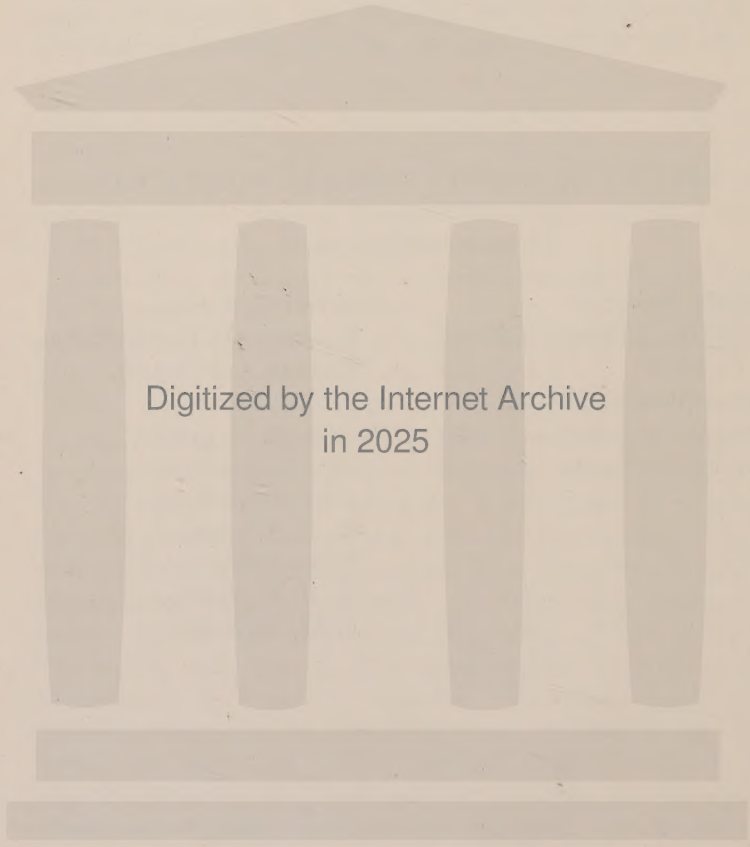
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To the memory of my friends and colleagues

Howard Taylor Ricketts

Richard Weil

Two pioneer American investigators in the field of immunology, who gave their lives, one in the service of science, the other in the service of his country. Through their early death, the progress of human knowledge and the welfare of their fellow men suffered an immeasurable loss.

AUTHOR'S PREFACE TO THE FIRST EDITION

Originally the reactions of immunity were studied with the purpose of solving urgent problems concerning the cure, diagnosis and prevention of disease. After a time there came to be a growing recognition of their importance as general biological phenomena not exclusively concerned with disease. For the most part their chemical significance was less appreciated, largely because they were observed as reactions to bacteria, blood serum and cells, all of which are such complex mixtures of unknown constitution that any chemical consideration of their behavior is entirely impossible. Perhaps the hypothetical presentation of the subject in the terms of the Ehrlich nomenclature, with pictorial conceptions which had no chemical significance, had some influence in satisfying many investigators that they understood the principles when they merely understood the hypothesis. As Dean has said in this connection, "Ignorance, however aptly veiled in an attractive terminology, remains ignorance." The early recognition by Bordet of the similarity of the reactions of immunity to the reactions of colloid chemistry probably failed to impress the rank and file of investigators in immunology because they were, at that date, unable to appreciate the significance of the colloid-chemical viewpoint, for lack of knowledge of this new field of chemistry.

In course of time, however, it began to be appreciated more and more that these reactions of immunity are important, not merely for their application to medical practice, but as general biological phenomena and as processes of biological and colloidal chemistry; therefore there has of late been more and more consideration of immunology from these standpoints. The progress towards an understanding of the fundamental principles has been slow, because in the study of the immunological processes we must have on one side of the equation a living animal and on the other the most complex of all known chemical compounds, the proteins or closely related colloidal materials. The only way in which we can simplify the equation is by using purified proteins, preferably those of as well-known composition as possible, in place of such hopelessly complex mixtures as bacteria or blood serum, and even then we have not obtained any very simple component. Undoubt-

edly knowledge will grow with the progress of colloidal chemistry, and such studies as those of Jacques Loeb on the behavior of protein solutions bid fair to throw more light into the knowledge of immunity than most of the direct investigations of immunological problems.

It would seem to be fitting, therefore, that a series of monographs covering modern chemistry include a discussion of immunity from the chemical standpoint, despite the evident lack of maturity of this new field of chemistry. The critical reader will appreciate that the chemistry of immunology has so far had but a fragmentary and illogical development—here, the work of an immunologist struggling with agents of unknown composition and measuring results with a yard stick of most uncertain accuracy; there, the efforts of a physical chemist applying methods of great accuracy to materials of uncertain nature and to reactions modified by an infinity of unknowable variables. No one can expect that from data so derived, in a new science in which the contributions of tomorrow contradict many of those of yesterday, any clear picture or final statement can be presented. At most, one can consider as much of the evidence as he can digest, present as much as seems necessary to carry the thread of the argument, and hope to convey a fair and impartial impression of how the matter stands now and in what direction the subject appears to be moving.

Since it is probable that such a presentation will have for its audience a mixed group of chemists and immunologists, their differing requirements have been kept in view. Presumably the chemist will wish to know to what extent immunology is a branch of chemistry. Works on immunology will be closed to him by the complex and vague terminology that has been developed because the lack of sufficient knowledge of the processes involved permits the use of no more exact terms. For his benefit an introductory chapter has been provided with the intention of reducing, as far as may be, the obstruction offered by this terminology. Also, each chapter is concluded with a recapitulation of the contents, which may serve to present the essential facts unobscured by the mass of evidence on which they are based. Probably the chemist who is seeking to familiarize himself with immunology will do best to read the recapitulation first, in order to be able to follow the line of argument in case he wishes for more details than the recapitulation furnishes.

The immunologist is entitled to expect that a monograph on the chemical aspects of immunity shall present a digest of the entire subject, so arranged as to constitute not only a summary of the situation, but

also to serve as an illustrated guide to the literature dealing with these chemical aspects. He will appreciate that the bulk of the literature is so great that even were it possible to consider it all, the actual mass would render obscure the essentials of the line of development. No attempt has been made to cite all the scattered literature, but such references as are cited will be found to furnish adequate bibliographies to cover practically all contributions of importance.

Many colleagues are entitled to my gratitude for their assistance in the preparation of this monograph. To remove the difficulties inherent in the attempt by one man to cover so wide a field, each chapter has been read over by at least one chemist and one immunologist, and most of them by several of each. It is hoped that through their help the number of actual errors of statement has been kept to a minimum and that serious omissions have not occurred. However, for such errors of commission or omission the final responsibility is mine, and by failing to give individual credit and thanks by name, I shall avoid the appearance of shirking this responsibility, without in the least decreasing my debt to my friends for their generous aid.

August, 1924. H. G. W.

AUTHOR'S PREFACE TO THE SECOND EDITION

The cordial reception received by the first edition of this book, evidenced by the exhaustion of the American edition and its publication in German and French translations, indicates an active interest in this newly developing field of the biological sciences. The opportunity is thus presented, and the responsibility recognized and accepted, for revising and bringing to a later date this analysis of a rapidly growing subject. As before, the effort has been made to cover, as briefly as is consistent with clarity and reasonable completeness, the facts and theories now available that bear on the chemical processes by which living creatures defend themselves against injury, and the various related chemical reactions of animal tissues to foreign substances. In the four years that have elapsed since the first edition was prepared there have been many contributions coming within the scope of this review, which have been considered as carefully as possible and incorporated in this revision to such extent as seems desirable for the purpose in view. The newer contributions on the influence of lipoids and carbohydrates on antigenic activity and specificity, the refining of antibodies to near the vanishing point, the chemical modification of antigens, the increasing study of isolated antigens, the growing recognition of the differences between complex and simple antigens, and the varied if unsuccessful attacks on the problems of specificity, have been outstanding among the many items taken into account and discussed in the present revision. Many colleagues have again most generously given of their time and knowledge in reviewing those portions of the manuscript dealing with topics with which they are particularly familiar, and my indebtedness to them all is hereby most gratefully expressed.

August, 1929. H. G. W.

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THE CHEMICAL ASPECTS OF IMMUNITY

CHAPTER I

INTRODUCTION

Immunological reactions, the processes by which the living organism defends itself against the chemical attacks of its enemies and so is able to exist in an environment seething with such enemies, are chemical reactions. The reagents involved are substances endowed with active chemical properties, and they are the product of chemical activity of the tissues of the body. In few if any cases do we know the chemical constitution of either the poison of the parasite or the defensive agent of the host, and our knowledge is gained entirely by observing the reaction or the effects resulting from the reaction. Therefore the chemistry of immunity is on quite the same plane as the chemistry of the enzymes, and our lack of exact knowledge, coupled with the vitally important nature of the processes concerned, makes all the more stimulating the investigation of the unanswered problems.

Despite the lack of any definite information as to the fundamental principles and agents of the immunological reactions, these reactions have already become of practical value in the study of many problems of chemistry. They permit us to determine the presence of infinitesimal amounts of proteins concealed in mixtures of great complexity, and to ascertain many facts about these proteins which can be determined accurately by no other known chemical means. They permit us to tell from just what species of animal a minute fleck of blood or scrap of meat has come; or whether a sample of meal contains other than the sort of plant material it is supposed to be. They give the protein chemist a method of determining the purity and many other facts concerning the preparation with which he is working, and with the expenditure of but trifling quantities of material. The physiologist may detect, by the methods of the immunologist, in the lymph coming from

the thyroid gland the presence of quantities of the specific thyroglobulin that are far too small to be detected by any known analytical or physiological method.[1] These reactions indicate that the chemistry of the blood proteins is different in even closely related species of animals, but that some other proteins, such as the crystalline lens of the eye or the albumin of the egg white, may be nearly or quite identical in widely separated species, these resemblances and differences often being extremely difficult of detection by any other chemical methods now available.

In view of these facts, the presentation of a recapitulation of our knowledge of the chemical aspects of immunity is deemed appropriate in a library of chemical monographs. However, such a presentation meets at the outset considerable difficulty, depending on the fact that immunology, in common with other subdivisions of biological science, has built up its own nomenclature. This nomenclature at once appears as a serious obstacle to the uninitiated reader, but it cannot be evaded. As with all special nomenclatures, the new terms have been devised out of necessity to permit of the expression in a single word or phrase what otherwise would require endless wasteful and tiring circumlocution. Therefore, in a work intended at least in large part to be of use to chemists and biologists not primarily engaged in the study of immunology, it seems necessary to present as a foreword a brief statement of the fundamental principles and descriptive definitions of the terminology employed in this field.[2]

The following paragraphs, therefore, offer definitions of the terms in most common use, with a brief statement of the fundamental principles involved.

Antigens.—As the word implies, antigens are substances which, when introduced into the body of an animal under proper conditions, stimulate this animal to produce substances which may combine specifically with these antigens; that is, antigens incite the formation of antibodies. Not all foreign substances, whether poisonous or not, have this capacity to incite the production of antagonistic substances; for example, alcohol, alkaloids, mineral poisons, sugars, are not antigens. Antigens seem always to be large colloidal molecules, and in general

[1] Hektoen, Carlson and Schulhof, *J. Am. Med. Assoc.*, 81:86 (1923).

[2] The principles and known facts of immunity will be found adequately presented in Zinsser's "Infection and Resistance," Macmillan; and in Kolmer's "Infection, Immunity and Specific Therapy," W. B. Saunders. The former is to be recommended especially for its discussion of principles, the latter for details as to methods. Those wishing to secure a working knowledge of immunology, and desiring statements more concise than are to be found in such complete treatises, are referred to Karsner and Ecker, "The Principles of Immunology," Lippincott.

nearly all soluble proteins are antigenic, there being some doubt as to whether anything except protein molecules can serve as antigens.

Antibodies.—These are the agents or qualities which appear in the blood of the immunized animal and exhibit the property of reacting specifically with the antigen used in immunizing. As antibodies may be found naturally in the blood in greater or less amounts before any immunization has taken place, it is customary to indicate such natural antibodies as normal antibodies, in contrast to the *immune* antibodies engendered by immunizing. As antibodies have never been isolated in a pure condition we have no knowledge as to what they are, and their existence is recognized merely by the effects they produce, just as we recognize the existence and presence of enzymes. The antibodies may be identified by their numerous different reactions, although as yet we do not know whether these several reactions are produced by one antibody or whether there are as many different sorts of antibodies as there are sorts of reactions by which they may be detected.

The most usual reactions employed in immunological work, and the terms used in discussing them, are the following:

Precipitin Reaction.—If the antigen is soluble it will, when added to the blood serum of the immunized animal (*antiserum*) in proper proportions, lead to the formation of a precipitate. The antibody concerned in this reaction is therefore called a *precipitin,* the antigen is called a *precipitinogen.*

Agglutinin Reaction.—If the antigen is not dissolved but is in the form of visible particles, e.g., bacteria, red corpuscles and other cells, these particles will, under the influence of the antiserum, adhere to one another to form flocculi which usually are then precipitated. If the cells are motile, e.g., typhoid bacilli, spermatozoa, they also lose their motility. In this reaction the antibody is called an *agglutinin* and the antigen is an *agglutinogen.* Evidently these two reactions (agglutinin and precipitin) are closely related, the differences depending entirely on the size of the colloidal complex serving as antigen, and there is much reason to believe that precipitins and agglutinins are identical.

Toxin-antitoxin Reaction.—If the antigen is poisonous the antibody may be capable of neutralizing it. A poisonous antigen which engenders such a specific immunizing substance is called a *toxin*; the antibody is an *antitoxin.* Poisonous substances which are not antigenic, i.e., such substances as morphine or arsenic, which do not incite the formation of specific antibodies, should not be called toxins. Some bacteria secrete or liberate into the fluids about them soluble toxins

which are able to engender antitoxins when injected into animals, but most of the pathogenic bacteria do not do so, although when their structure is broken down poisonous materials which are not antigenic are often released. These two types of poisons are distinguished by the names *exotoxin,* for the true soluble antigenic poison secreted by such bacteria as *B. diphtheriæ* and *B. tetani;* and *endotoxin* for the intracellular, non-antigenic poison which is liberated only on disintegration of the bacteria. There are numerous antigenic poisons besides bacterial toxins which engender specific antitoxins on immunization, such as snake venoms and the plant toxins (ricin, abrin, etc.). When the toxins are modified by physical or chemical means so that they lose their toxicity but retain their antigenic capacity to incite antibody formation, they are called *toxoids.*

Anaphylaxis.—If an antigen is injected into an animal, the animal may, after seven days or more of incubation, show a marked *hypersensitivity* to this antigen, so that a very minute amount may intoxicate it severely or fatally, even if the antigen, e.g., egg white, was entirely nontoxic before the animal had been *sensitized* by injection of the first or sensitizing dose. This reaction was designated by Richet as anaphylaxis, indicating that the condition is the opposite of prophylaxis. This and all other forms of altered reaction to antigens, and even to nonantigenic substances, are sometimes grouped together under the term *allergy,* meaning altered reactivity. The antibody invoking the anaphylaxis reaction is usually called *anaphylactin* or *allergin,* although the term *sensibilisin* has also been employed, the antigen being *anaphylactogen,* or *allergen,* or *sensibilisinogen.*

Lysis.—In case the antigen is in particulate or cellular form, the action upon it of immune serum may lead to its solution, the process being called lysis, and lysis of special forms of antigens is appropriately indicated by such terms as *bacteriolysis, cytolysis, hemolysis,* etc. Dissolved antigens may also be disintegrated by immune serum (*proteolysis*). The antibody concerned is called a *lysin,* but it is known that in addition to this specific antibody, engendered by immunization with the specific antigen, there is required another agent to complete the lytic action. This second component is present in the normal serum of unimmunized animals as well as in immune serum, and as it complements the action of the antibody, or lysin, it has been called *complement.* The term *alexin,* meaning protective substance, has also been employed, since it is an important factor in protecting against infection. As the immune antibody or lysin involved in this type of reaction has

been pictured as uniting the complement to the antigen, it has been called an *intermediary body;* and also, because it was supposed to have an affinity or receptor for the antigen and one for the complement, Ehrlich christened it *amboceptor.* Other terms have also been used, but the above are most generally employed.

Numerous other phenomena of immunity, which introduce still other terms, will be discussed in their proper place, but the foregoing statements cover the best known reactions and most used terms, and hence will suffice for an introduction.

CHAPTER II

ANTIGENS

By definition an antigen is any substance which, introduced into the tissues or circulating fluids of an animal, causes the appearance in these fluids, sooner or later, of substances which react specifically with the antigenic substance; i.e., specific antibodies. This definition may possibly be a trifle narrow, for we may imagine specific antibodies being produced and remaining within cells, not becoming demonstrable in the circulating fluids. In artificial cultures of tissue cells, also, specific antibodies may be formed in reaction to the presence of antigens when there is no circulating fluid. But practically we cannot well determine that a given substance is antigenic unless its injection into the body of an animal in suitable quantities and under suitable conditions leads to the production of antibodies demonstrable in the blood by the reactions they exhibit in the presence of the specific antigen.[1]

Certainly the word antigen clearly means an agent that engenders antibodies, but sometimes the converse assumption is made, viz., that a substance which reacts with a specific immune serum is an antigen. While it is true that antigens react with antibodies, it does not necessarily follow that everything reacting with antibodies is antigenic. There are many substances, especially lipoids and certain complex carbohydrates, which are not themselves antigenic but which may react more or less specifically with antibodies under certain conditions. Many authors refer to such substances as antigens, which practice has led to much confusion. This matter is discussed more in detail later. (See p. 39.)

To exhibit its function the antigen must penetrate beyond the epithelial surfaces of the body, and the assumption is naturally made that the development of antibodies is a defense against the presence within the tissues and fluids of the body of proteins foreign to the organism, for only foreign proteins are ordinarily antigenic. The epithelium of cutaneous, alimentary, and probably also placental sur-

[1] The chief exception is the existence of antibodies bound in the non-striated muscles which may be demonstrated by the reactivity of such muscles to the specific antigen (anaphylaxis).

faces, is an almost perfect barrier to the penetration of foreign proteins, as illustrated by the fact that such toxic foreign proteins as those of snake venom are harmless when taken into the alimentary canal. This protective action of surface epithelium is not exhibited by the deeper tissue cells with which an injected foreign protein comes in contact, and the immunological reactions seem to constitute a secondary line of defense (Grosser).[2]

The Character of Antigens

For the most part antigenic substances are proteins. At the present time it has not been conclusively established that anything except proteins ever exhibits true antigenic activity and leads to the production of specific antibodies. On the other hand it is safe to state that nearly, if not every known sort of soluble protein occurring in nature is antigenic, if we limit our use of the term protein to those colloidal aggregates of amino acids which contain the full quota of amino acids usually found in "complete" proteins.

Of course, if a protein is not soluble in the plasma of an animal it cannot reach the sites of antibody production and therefore can exhibit no antigenic activity. Hence proteins that have been irreversibly coagulated by heat commonly exhibit no antigenic activity, but proteins which are not coagulated by boiling (e.g., casein) retain their antigenic activity unimpaired after being subjected to this degree of heat, indicating that it is not the effect of alteration in the protein molecule induced by heat that destroys antigenic activity unless these alterations lead to loss of solubility.[3]

Antigenic activity is not dependent on toxicity, for many highly antigenic proteins are of low toxicity, and toxins that have been detoxicated by various means (toxoids, anatoxins) are still strongly antigenic. Combination of antigen with antibody may reduce the antigenic activity,[4] but does not destroy it, perhaps because the antigen slowly dissociates from the antibody after injection, as in toxin-antitoxin immunization.

As yet we do not know to what the proteins owe their antigenic activity. Gelatin, which is not a naturally existing protein, but one derived by hydrolysis of the insoluble connective tissue protein, collagen,[5] ex-

[2] *Anat. Anzeiger*, **53**:49 (1920).
[3] Wells, *J. Infect. Dis.*, **5**:449 (1908); *J. Biol. Chem.*, **28**:11 (1916).
[4] See Iijima, *J. Path. Bact.*, **28**:397 (1925).
[5] Glue, which is closely related to gelatin, is also devoid of antigenic activity [Ramsdell and Walzer, *J. Immunol.*, **14**:207 (1927).]

hibits no demonstrable antigenic effect.[3, 6, 7, 8] As gelatin is characterized chemically by its lack of aromatic radicals, for it contains no tryptophan or tyrosine and but a small amount of phenylalanine, the inference seems warranted that the aromatic radicals of the protein molecule are of importance in determining antigenic activity. In support of this idea are the following facts:

(1). Vaughan [9] showed that when toxic and non-toxic fractions are formed from proteins by cleavage, the toxic fraction contains the aromatic radicals, and gelatin does not yield a toxic fraction when subjected to such cleavage. If the immunological reactions are defense reactions it is to be expected that the toxic character of the protein or its cleavage products will be a determining factor in the stimulation of antibody formation.

(2). Proteins possessing a full complement of aromatic radicals but deficient in some of the other amino acids commonly found in proteins, exhibit active antigenic properties. Among such proteins are the following: Zein, which lacks tryptophan, lysine and glycine, but possesses a large proportion of tyrosine; gliadin, which lacks lysine; egg albumin, which lacks glycine; casein, which lacks cystine and glycine.

(3). The protamines,[9a] which consist chiefly of complexes of diamino acids with but a small total quantity of a few of the mono-amino acids of proteins, are devoid of antigenic activity.[10, 11] This fact also suggests that the diamino acids are not of importance in respect to antigenic function, an assumption which is supported by the antigenic activity of hordein from barley, which protein contains no lysine and very little arginine or histidine. As no proteins are known which do not contain either histidine or arginine we cannot prove their lack of essential antigenic significance as we can for lysine.

(4). Obermayer and Pick [12] and others have found evidence that the aromatic radicals of the proteins may be of importance in determining the specific character of immunological reactions (see Specificity, Chapter III).

Undoubtedly an important and perhaps essential factor in the antigenic activity of proteins is their large molecular dimension with the attendant colloidal properties. Whenever the protein molecule is broken

[6] Wells, *J. M. Med. Assoc.*, **50**:527 (1908).
[7] Starin, *J. Infect. Dis.*, **23**:139 (1918); Landsteiner, *Z. Immunität.*, **26**:152 (1917).
[8] Kahn and McNeil, *J. Immunol.*, **3**:277 (1918).
[9] V. C. Vaughan, "Protein Split Products," Philadelphia, 1913.
[9a] See Kossel, "The Protamines and Histones," Engl. Transl., Longmans, Green Co., New York, 1928.
[10] Wells, *Z. Immunität.*, **19**:599 (1913).
[11] Schmidt and Bennett, *J. Infect. Dis.*, **25**:207 (1919).
[12] *Wien. klin. Woch.*, **19**:327 (1906).

down into smaller fragments it loses its antigenic properties *pari passu*. Zinsser [13] suggests that antigens must be non-diffusible colloids, which therefore cannot enter the cells to be destroyed therein, so that it becomes necessary for soluble, extracellular antibodies to be formed which may react with the foreign protein as a step in its destruction. When the foreign molecule is small enough to be taken into the cells by diffusion, antibody formation becomes unnecessary for its destruction, and consequently we have no antigens that are not colloidal. In support of this is the fact that the cleavage products of a protein, even when injected all together, have no antigenic capacity, although when in their original combined colloidal form they are antigenic, and when artificially reunited into colloidal molecules (called plasteins) the antigenic capacity of these cleavage products may be restored. Also, ultrafiltrates of bacterial suspensions, being devoid of colloids, are not antigenic.[14]

The antigenic activity, as measured by the amount or rapidity of antibody production, varies much with different proteins. Sometimes this seems to depend on the solubility of the antigen in the tissue fluids, as in the case of the vegetable proteins; [15] but, in other cases, no such explanation can be found. For example, serum albumin seems to be much less strongly antigenic than the globulins from the same serum.[16] Relative antigenic activity cannot be correlated with the *size* of the protein molecules, at least if the present assumptions as to the molecular weights of protein are correct, since egg albumin (a good antigen), and serum albumin (reputedly a poor antigen) have relatively low molecular weights,[17] while high weights are assigned to euglobulin (a good antigen) and casein (a poorer antigen).

With any given protein the amount of antibody formation does not vary directly with the amount of antigen injected, small doses often producing even larger amounts of antibody than larger doses.[18] As a rule, the same amount of antigen in divided doses at intervals of a few days, provokes a greater antibody formation than if injected at one time, but this is not always true.

There is a great individual variation in the antibody production by different animals, even of the same species, in response to the same amount of antigen. Part of this difference seems to depend on definite differences in the serology of the individual animals. For example, some

[13] "Infection and Resistance," p. 110. 1923.
[14] Fernbach and Hepner, *Centr. Bakt.*, 1 *Abt.*, 100:358 (1926).
[15] Wells and Osborne, *J. Infect. Dis.*, 14:377 (1914).
[16] Dale and Hartley, *Biochem. Jour.*, 10:408 (1916); Doerr and Berger, *Z. Hyg.*, 96:191 (1922); Ruppel, *Deut. med. Woch.*, 49:40 (1923).
[17] See Cohn, *Physiol. Reviews*, 5:349 (1925).
[18] Tsen, *J. Med. Research*, 37:381 (1918).

rabbits naturally possess antibodies for one of the specific groups of human red corpuscles, and only these rabbits produce specific antibodies on immunizing with this group of corpuscles (Dölter and Witebsky).[19]

Falk [20] found evidence that the pH of the protein solution used for immunizing has some influence on the amount of immunological response, acid solution (pH = 2.5) generally being more effective than alkaline (pH = 10), but these effects are not constant.

Apparently incredibly minute amounts of material may serve as antigen; thus, Seibert [21] found that immunization with the traces of solid material present in 20 cc. of non-sterile distilled water produces agglutinins active in serum dilutions of 1 to 8000 against the bacteria growing in such distilled water, although the protein content of the injected water was only about 0.0001 gm. Branham and Humphreys [22] have obtained potent antisera by immunizing with filtrates from *B. enteritidis* which gave no protein reactions, but did react faintly for tryptophan and histidine, the amount of protein being estimated at below 0.000,003 gm., if any was present at all. Hartley [23] has obtained diphtheria toxin-antitoxin precipitates that were actively immunizing in doses containing less than one ten-thousandth of a milligram of nitrogen. Such results indicate the possibility of the existence of bacterial antigens that are not proteins, but diphtheria anatoxin, which behaves like a protein, has been prepared with an immunizing action in a dose of 0.000,05 gm.[24]

Can Homologous Proteins be Antigenic?

Under ordinary conditions the proteins native to an animal (*homologous proteins*) do not stimulate any antibody formation in that animal. Obviously, if they did, every animal would be producing antibodies against its own components, which would lead to a situation dramatically designated by Ehrlich as *"Horror autotoxicus."* But apparently under certain conditions homologous proteins may become so altered as to behave as foreign proteins. For example, Doerr [25] cites instances in the literature in which persons have given typical anaphylactic reactions to repeated injections of human blood or serum, and various authors have claimed that repeated injections of the blood, serum or tissue extracts from one animal into another of the same species has

[19] Quoted by Sachs, *Centr. Bakt.*, Beiheft, 104:138 (1927).
[20] *J. Immunol.*, 8:239 (1923); *J. Infect. Dis.*, 37:514 (1925).
[21] *Amer. J. Physiol.*, 71:621 (1925).
[22] *J. Infect. Dis.*, 40:516 (1927).
[23] *Brit. J. Exp. Path.*, 7:55 (1926).
[24] Koulikoff and Smirnoff, *Ann. Inst. Pasteur*, 41:1166 (1927).
[25] *Ergeb. Hyg., Bakt., Immunität. u. exp. Ther., Berlin*, 5:137 (1922).

led to the production of antibodies (*iso-antibodies*). However, attempts to produce iso-antibodies are usually unsuccessful, and when successful the activity of the antisera is low. If antigenic activity depends entirely on parenteral digestion it is perfectly possible for homologous proteins to be antigenic, for we find that whenever tissues die they undergo spontaneous proteolysis. Fibrinous exudates and extravasated blood undergo digestion and absorption, and, according to Abderhalden, all such digestion of homologous tissues and proteins leads to the appearance in the blood of an increased capacity to digest the same particular kind of protein, which he attributes to the presence of specific defensive proteolytic enzymes (*Abwehrfermente*). (See Chapter VII.)

When an animal's proteins are chemically altered they may also behave as foreign proteins and lead to auto-antibody formation. For example, if the serum of an animal is treated with formaldehyde, nitrous acid, iodin, or numerous other substances which combine with the proteins, this altered serum may serve as an antigen and give rise to the production of specific antibodies when injected into the body of an animal of the same species from which it was obtained. Probably some of the tissue proteins of an animal may be foreign to the other tissues of the same animal if they escape into the circulation. Thus, the protein of the crystalline lens is said to serve as antigen in animals of the same species (see page 71), but if true, this iso-antibody formation is not usually marked.[26]

The Effect of Alterations in the Protein Molecule on the Antigenic Property

A. **Coagulation.**—Complete irreversible coagulation of proteins would, of course, render them non-antigenic, by preventing them from reaching the site of antibody formation in solution. If the coagulation is reversible, however, the redissolved protein is still actively antigenic if it has not been too much changed in composition during the processes of coagulation and resolution. Thus, if serum is coagulated by strong alcohol it can be redissolved in water after the alcohol has been removed, and the dissolved serum is antigenic.[27] Egg albumin, however, is practically irreversibly coagulated by alcohol, and the coagulated protein is in corresponding degree devoid of antigenic capacity.[28] As a matter

[26] Hektoen, *J. Infect. Dis.*, 31:72 (1922).

[27] Horse meat protein seems to become completely insoluble, at least it loses its antigenic power, if exposed long enough to strong alcohol—60-120 days, according to Kodama [*Z. Hyg.*, 74:30 (1913)].

[28] Wells, *J. Infect. Dis.*, 5:449 (1908).

of fact the coagulation even of egg albumin by heat is not absolutely irreversible, and to just the extent that a suspension of coagulated egg albumin redissolves it exhibits antigenic activity. I have found that 5 cc. of a suspension of thoroughly washed heat-coagulated egg albumin, which has stood for some months in salt solution, may contain enough redissolved egg albumin to sensitize occasionally, but not always, a guinea pig so that it will give an anaphylactic reaction with egg albumin.[29] As the minimum sensitizing dose of egg albumin is not far from 0.000,000,05 gram, it is evident that the solubility of coagulated egg albumin is extremely low. Experiments with the delicate complement fixation test establish similar values for the amount of free egg albumin dissolved in water in which coagulated egg albumin has been suspended for several weeks or months.[30]

These figures give a striking illustration of the delicacy of the immunological methods and their value in studying certain problems in protein chemistry. In no other way could such minute amounts of protein be detected in a solution. Furthermore, the immunological tests not only disclose the presence of this minute amount of protein in solution, but specificity tests establish also that it is the same protein that was coagulated by heat which has been redissolved in its original form and not merely a product of hydrolysis of the coagulated protein. Chemical tests on relatively large amounts of such fluids might possibly disclose that amino compounds, perhaps even proteins or their cleavage products, are present in the solution, but they could not possibly establish the character of the dissolved molecules as intact molecules of egg albumin, a feat easy of accomplishment by the immunological tests.

Proteins that are not coagulable by heat do not seem to suffer appreciably from boiling, at least under conditions that do not lead to hydrolysis. However, there seem to be few complete proteins that do not undergo coagulation on boiling in solutions at or near the neutral point. Those that have been investigated are found to retain their antigenic properties after brief boiling, these being: casein,[28, 31] ovomucoid,[32, 33] the so-called proteoses of plant seeds,[34] mucins and sero-

[29] Anson and Mirsky [*J. Gen. Physiol.*, 9:169 (1925)] also find that denaturization of hemoglobin can be reversed, and the specificity, lost in the denatured protein, is restored in the regenerated hemoglobin. They believe denaturation of proteins to be truly a reversible process, but ordinarily masked by the flocculation of the denatured protein.

[30] Zinsser [*J. Immunol.*, 9:227 (1924)] found that the filtrate from boiled serum or egg albumin (once crystallized) gives a precipitin reaction with strong antisera for these antigens. This merely proves that heat coagulation is not a complete process with such proteins (which undoubtedly contain noncoagulable mucoids) and has no bearing on the question of reversibility of the protein coagulation.

[31] Wells, *J. Am. Med. Assoc.*, 50:527 (1908).

[32] Wells, H. G., *J. Infect. Dis.*, 6:506 (1909).

[33] *Ibid.*, 9:147 (1911); Lewis and Wells, *ibid.*, 40:316 (1927).

[34] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 17:259 (1915).

mucoids,[35] and beta-nucleoproteins.[36] Of these, however, only the plant "proteoses" possess strong antigenic properties, although casein is far more active than some authors have maintained.[37] If heat coagulable proteins are boiled under conditions that prevent their coagulation (e.g., much diluted or at an unsuitable pH), they may retain some antigenic activity, but their specificity may be altered.[38]

Bacterial proteins seem to retain their antigenic capacity after being exposed to strong alcohol or to 1 per cent osmic acid.[39] On the other hand, red corpuscles, or suspensions of their stroma, are reduced in antigenic capacity after treatment with osmic acid.[40]

Ultraviolet light reduces the antigenic activity of proteins,[41] apparently affecting them in much the same way as heat, for coagulable proteins are converted into an irreversibly precipitated material when exposed under suitable conditions to strong ultraviolet radiation,[42] or, if not coagulated, they form modifications that are readily precipitated by various salts [43] and show other changes indicating decreased colloidal lability.[44] Even sunlight from which infra-red and ultra-violet rays have been removed, causes changes similar to those of heat coagulation.[45] Ultraviolet light acting on meningococci alters somewhat their immunizing properties (Eberson).[46]

B. **Cleavage.**—Free amino acids are not antigenic. In the hydrolytic cleavage of proteins the power to invoke antibody formation is lost somewhere between the intact stage of the protein molecule and its complete separation into the constituent amino acids, but as yet we do not know just where. Although there have been many contradictory findings reported by various immunologists as to the antigenic capacity of proteoses, peptones and peptids, most of the statements in the literature have evidently been made with little comprehension of the changes involved in protein hydrolysis or of the nature of the mixtures under investigation. Much of the recorded work has been carried out with commercial preparations of protein cleavage products, such as Witte's "peptone" and the like. These are notoriously variable preparations,

[35] Elliott, C. H., *J. Infect. Dis.*, **15**:501 (1914); Goodner, *ibid.*, **37**:285 (1925); Lewis and Wells, *ibid.*, **40**:316 (1927).
[36] Wells, *J. Biol. Chem.*, **28**:11 (1916).
[37] Wells and Osborne, *J. Infect. Dis.*, **29**:200 (1921).
[38] See Furth, *J. Immunol.*, **10**:777 (1925); **11**:215 (1926); Wu, Tenbroeck and Li, *Chinese J. Physiol.*, **1**:277 (1927).
[39] Thorsch, *Biochem. Z.*, **66**:486 (1914).
[40] A. F. Coca, *Biochem. Z.*, **14**:125 (1908); A. von Szily, *Z. Immunität.*, **3**:451 (1909); Kosakai, *J. Path. Bact.*, **23**:425 (1920).
[41] Doerr and Moldovan, *Wien. klin. Woch.*, **24**:555 (1911).
[42] Schanz, *Biochem. Z.*, **71**:406 (1915); *Pflügers Arch.*, **170**:646 (1918).
[43] Burge, *Am. J. Physiol.*, **39**:335 (1916).
[44] Mond, *Arch. ges. Physiol.*, **196**:540 (1922).
[45] Young, *Proc. Royal Soc. (London)*, **93B**:235 (1922).
[46] *J. Immunol.*, **5**:345 (1920).

concerning the source, composition and purity of which the observer has no knowledge. To call work done with such materials scientific investigation is, in my conviction, a prostitution of the word science, a parody of its standards and ideals. To make matters worse, much of the published evidence as to the antigenic value of protein fragments has been obtained with the anaphylaxis reaction. Here the criteria of reaction are furnished by the behavior of an injected animal, and as many of the symptoms of the anaphylaxis reaction (q.v.) may be essentially reproduced by intoxication with protein cleavage products independent of any immunological reaction, the value of the evidence becomes dubious indeed.

A critical review of the literature and a re-investigation of the subject in this laboratory by Fink [47] emphasizes the worthlessness of much of the published evidence on this subject. He concluded that positive, corroborated evidence of the production, by immunization with proteoses or peptones, of precipitins and complement fixing antibodies, the presence of which can be determined objectively, had not been presented. Fink himself studied the proteose fractions obtained by hydrolyzing coagulated egg white with steam under pressure and did find some evidence of slight antigenic activity by means of complement fixation, precipitin and anaphylaxis reactions in those fractions precipitated by three-fourths and full saturation with ammonium sulfate, but not with fractions precipitated at ¼, ⅓, ½ and ⅔ saturation. Of course we do not know just what is in these several fractions that may be obtained by salting out the products of protein cleavage. It might be expected that the largest molecules would come out in the precipitates first obtained with the smallest concentration of the salt, and that these should be more like the unhydrolyzed protein molecules. Fink's results, which seem to be definite, although slight, are therefore surprising in revealing that the first fractions contain no antigenic molecules, these appearing only in the last fractions. They emphasize our ignorance of the steps by which protein molecules undergo hydrolysis, and suggest another aspect of protein chemistry in which immunological methods may prove of value. There is no reason at present to assume that the so-called proteoses and peptones of various designations represent anything but the crudest of mixtures,[48] or to expect that constant results will be obtained in any investigation as to their properties, whether immunological or something else.

[47] *J. Infect. Dis.*, 25:97 (1919).
[48] See Haslam, *J. Physiol.*, 32:267 (1905); 36:164 (1907).

If no positive results can be obtained with most of the fractions of protein cleavage, including complete digestion mixtures of various proteins containing usually all the cleavage products, from proteoses through peptones and polypeptids to amino acids, it is not easy to accept the statement that anaphylaxis can be produced by synthetic polypeptids, even one containing 14 molecules of leucine and glycine (Abderhalden),[49] still less the positive results of Zunz[50] with much simpler polypeptids (3-5 glycylglycine). These reactions were obtained after intravenous injections of the peptids into sensitized rabbits, and consisted of fall of blood pressure, increased rate of respiration and expulsion of feces and urine, but these reactions, slight as they are, were by no means constant, and occurred under very particular conditions, e.g., positive results with animals sensitized with six intraperitoneal injections at weekly intervals, but not in animals that received six subcutaneous injections at similar intervals. Certainly, in view of the possible sources of such reactions independent of true anaphylactic shock, and the large number of recorded negative results with peptones and digestion mixtures, the acceptance of the results yet obtained with synthetic polypeptids as proof of their antigenic activity will require much more convincing evidence than any that has yet been produced.

Plasteins.—On the other hand, if digestion products are resynthesized into intact protein molecules they then may exhibit antigenic properties. At least this is the case if we accept the statement that the so-called "plasteins," formed supposedly from proteoses by synthesis through the reverse action of proteolytic enzymes, are true synthesized proteins.[51] Gay and Robertson[52] found that although the pepsin digest of casein is not antigenic, the plastein which they call paranuclein and believe to be formed by resynthesis of a protein through the action of pepsin on the casein digest, was capable of sensitizing guinea pigs to itself and to casein; rabbits immunized with paranuclein produce a serum giving complement fixation reactions with paranuclein but not with casein, and precipitin reactions with neither. Landsteiner[53] also prepared a "plastein" by the action of rennin on Witte peptone, which produced an immune serum giving precipitin reactions with the plastein as well as with the Witte peptone.

[49] *Z. physiol. Chem.*, **81**:315 (1912).
[50] Zunz, E., *Biochem. J.*, **10**:160 (1916); *J. physiol. Path. gén.*, **17**:449 (1917); *Arch. Internat. physiol.*, **15**:79, 92 (1919).
[51] This seems to be established by the work of Wasteneys and Borsook, and of Beard [*J. Biol. Chem.*, **71**:477 (1927)].
[52] *J. Biol. Chem.*, **12**:233 (1912).
[53] *Biochem. Z.*, **93**:106 (1919).

Hermann and Chain [54] found that a plastein made from Witte peptone engendered a precipitin which reacted with the same plastein, also with plasteins from unrelated sources (proteoses from edestin, serum or egg albumin, almond globulin), but not with the original peptone or protein from which the plastein was derived. A similar lack of specificity was observed with plasteins investigated by v. Knaffl-Lenz and Pick,[55] who obtained precipitins but were unable to produce anaphylaxis, either active or passive, with their plasteins.

Compound Proteins as Antigens

Addition of elements or radicals to antigenic protein molecules seems not to impair their antigenic activity provided the resulting compound is still soluble. Casein, which is a phosphoric acid salt of a protein, is an effective antigen.[56] Mucins, which seem to be compounds of protein with chondroitin sulfuric acid,[57] or mucotin-sulfuric acid, are antigenic,[58] as also are the so-called beta-nucleoproteins [59] in which the non-protein radical is guanylic acid. Ovo-vitellin, which, like casein, is a phospho-protein, and ovomucoid, a glycoprotein, are also antigenic.[60]

Artificial Compound Proteins

Soluble artificial compound proteins, such as iodized and diazotized proteins,[61, 62] are also antigenic, although the specificity (q.v.) is altered by such chemical changes. There is evidence that the entrance of a new radical into a protein molecule may give it new properties that convert it into an immunologically different protein. This seems to explain certain cases in which an individual becomes hypersensitive to some simple chemical substance, not antigenic of itself, and reacts to this substance with the allergic phenomena characteristic of anaphylaxis, because the chemical has combined with some of the body proteins and thus formed proteins that are sufficiently foreign in character to incite antibody formation. In support of this conception is the fact that many of the chemicals that produce such specific reactions are chemicals that might readily unite with protein molecules and produce foreign compound proteins, e.g., iodin, arsenic, mercury, formaldehyde,

[54] *Z. physiol. Chem.*, **77**:289 (1912).
[55] *Arch. exp. Path. Pharm.*, **71**:298, 407 (1913).
[56] Wells and Osborne, *J. Infect. Dis.*, **29**:200 (1921).
[57] Levene, "Hexosamines and Mucoproteins," Monographs on Biochemistry, Longmans, London. 1928.
[58] Elliott, *J. Infect. Dis.*, **15**:501 (1914).
[59] Wells, *J. Biol. Chem.*, **28**:11 (1916).
[60] Wells, *J. Infect. Dis.*, **9**:147 (1911).
[61] Obermayer and Pick, *Wien. klin. Woch.*, **19**:327 (1906).
[62] Wells, *J. Infect. Dis.*, **5**:449 (1908).

salicylic acid. Landsteiner has shown that rabbit serum treated with formaldehyde, when injected into rabbits, causes the production of an antiserum which will give precipitin reactions with this formaldehyde rabbit serum, and not with the formaldehyde treated sera of other species.[63] Positive results have also been obtained with the anaphylaxis reaction used as the means of demonstrating antigenic activity.[64] It is to be remembered that formaldehyde is a substance which notoriously leads to a specific hypersensitive state, many pathologists and others who work with formalin exhibiting a most marked sensitivity thereto. Similar effects have been obtained with nitrated proteins (Obermayer and Pick[61]).

Klopstock and Selter[65] have found that merely mixing some of these sensitizing chemicals with proteins is sufficient to make them antigenic for animals of the same species as the one furnishing the protein. For example, diazotized atoxyl mixed with guinea pig serum if injected immediately into guinea pigs is not antigenic, but if the mixture stands for some time before injection it is then antigenic, and the injected animal is sensitized to the diazotized atoxyl. If the diazotized atoxyl is injected by itself intravenously into a guinea pig, no sensitization takes place, apparently because the atoxyl is removed from the blood before long enough contact with blood proteins has occurred; but subcutaneous injection produces sensitization, since in this case the contact is longer, and the animal then gives violent local reactions to subsequent injections of the atoxyl even without any accompanying protein. Presumably the crystalline substance isolated from primroses by Bloch and Karrer,[66] which produces primrose hypersensitivity, acts in the same way.

The properties of chemically changed proteins have been investigated by Obermayer and Pick, and those of artificial compound proteins especially by Landsteiner[67, 68] who found that proteins united to various simple radicals, especially nitrogenous radicals, when injected into animals induce the production of antibodies which give precipitin reactions with any and all sorts of similarly treated proteins, no matter whether the proteins were related in origin to one another or not. For example, the serum of a rabbit immunized with horse serum, the proteins of which have combined with metanilic acid (m-aminobenzolsulfonic acid),

[63] Landsteiner and Jablons, *Z. Immunität.*, **20**:618 (1914).
[64] Landsteiner, *J. Med. Res.*, **39**:631 (1924).
[65] *Centr. Bakt.*, **104**: Beiheft, p. 140 (1927); *Z. Immunität.*, **55**:118, 450 (1928).
[66] *Vierteljahrsch. Naturforsch. Ges. Zurich*, Beiheft, **72**:No. 13 (1927).
[67] *Biochem. Z.*, **93**:106 (1919).
[68] *Biochem. Z.*, **104**:280 (1920).

gives strong precipitin reactions with other metanilic acid protein compounds, even when the protein radicals are as unrelated to horse serum proteins and to each other as gliadin, globin, mucin, casein, legumin, silk, and rabbit serum.

Azotized albumoses give but slight precipitin reactions with an antiserum for azotized proteins, and azotized peptones and amino acids none at all, but such azotized compounds do possess the capacity to unite with the antibodies present in the serum, even when they do not produce precipitin reactions with this serum. Apparently, then, these small, non-colloidal molecules share with the azo-proteins the capacity to react with the immune bodies of the serum, but fail to produce a precipitate. This assumption is supported by the fact that even simpler reactive substances possess the capacity, when in excess, to inhibit the precipitin reaction between antibodies and antigens containing these radicals, this inhibition being specific for the compound used in combining with the protein that served as antigen in producing the immune serum.

The explanation advanced is that the inhibiting substances contain a group which is identical with the specific group of the derived protein which reacts with the immune serum. This group can therefore unite with the antibodies of the immune serum quite as well as can the same group when attached to protein, and hence if there is an excess of the simple chemical compound it will bind most of the antibodies and prevent the precipitin reaction with the derived protein. Such simple radicals are not antigenic in the sense of being able to incite in animals the production of antibodies with which the chemicals react; only when bound in a protein molecule used in immunizing do they have the effect of determining the specificity of the resulting antibodies. They do exhibit one attribute of an antigen, namely, reacting specifically with antibodies, but this quality by itself does not constitute an antigen.[69] It requires the large colloidal molecule of the proteins to stimulate the production of specific antibodies—which is the characteristic property of antigens.[69a] That any other large colloidal molecules than protein can serve as antigens when combined with a specific reactive group has not yet been determined, but Landsteiner suggests that such a non-protein antigenic complex is a possibility.

[69] Substances of this class are called *haptens* by Landsteiner. Similar substances, non-antigenic but capable of reacting specifically with immune antibodies, have been found in bacteria by Zinsser ("Infection and Resistance," p. 110, 1923), and others.

[69a] While the simple radicals used in Landsteiner's experiments resembled antigens only to the extent of inhibiting specifically the antigen-antibody reactions, similar radicals added to lecithin (diazo-atoxyl-lecithin) may give specific complement fixation with a diazo-atoxyl antiserum (Klopstock).

Protein-Free Carbohydrate "Antigens" (Haptens)

The foregoing facts may furnish the explanation of certain antigens obtained from bacteria which are said to be protein-free. Pick [70] found in young typhoid cultures a material that did not give the ordinary protein reactions, which was resistant to heat and proteolytic enzymes and soluble in alcohol; it exhibited the power of giving specific precipitin reactions with immune serum, but had no capacity to produce antibodies when used for immunizing. Zinsser and Parker [71] found in pneumococci, influenza bacilli and staphylococci, a similar substance, free from gross amounts of proteins, precipitated by alcohol, and thermostable, which gave specific precipitin reactions with homologous antiserums but produced no antibodies when injected into animals.[71a] This carbohydrate "hapten" will also produce anaphylactic reactions in animals previously sensitized to the whole cells, although it will not sensitize the animals.[72]

The nature of this hapten was made clear when Heidelberger and Avery [73] isolated from Type II pneumococcus cultures a material reacting specifically with antiserum for pneumococci, which appears to be a polysaccharide built up of glucose molecules, and is probably related to the gums found in numerous other capsulated bacteria. This material seems to be responsible for the type specificity of the reactions between pneumococci and antipneumococcus serum, but it appears to be incapable of stimulating the production of antibodies when used for immunizing, and therefore it is not a true antigen.[74] It seems to be similar to a complex carbohydrate isolated from yeast by Mueller and Tomcsik,[75] which reacts with precipitins for yeast although not of itself antigenic. Probably similar components can be found in any and all sorts of bacteria in which sought.

In the case of type I pneumococci the gum appears to be made up of sugar units which contain nitrogen as a part of the molecule. The gum itself contains 5 per cent N, half of which is liberated by HNO_2 with a loss of specific properties, and it may be composed of an amino-sugar derivative linked to galacturonic acid. It behaves as a strong acid and a weak base, with an iso-electric point at about pH 4, at which

[70] *Beitr. chem. Physiol.*, 1:397 (1902).
[71] *J. Exp. Med.*, 37:275 (1923); 42:311 (1925).
[71a] Similar non-protein haptens have been found in other bacteria, e.g., streptococci [Lancefield, *J. Exp. Med.*, 42:377, 397 (1925)]; cholera vibrios [Landsteiner and Levene, *ibid.*, 46:213 (1927)].
[72] Tomcsik and Kurotchkin, *J. Exp. Med.*, 47:379 (1928).
[73] *J. Exp. Med.*, 38:73 (1923); 42:347, 727 (1925).
[74] *See* review by Zinsser and Mueller, "The Newer Knowledge of Bacteriology and Immunity," Univ. of Chicago Press, p. 721, 1928.
[75] *J. Exp. Med.*, 40:343 (1924).

reaction it is fairly insoluble. The type III compound is produced in larger quantities than the others, and appears to be a soluble salt of an insoluble acid. It is hydrolyzed to a disaccharide acid which in turn splits into glucose and glucuronic acid.[75a] The optical properties of these substances vary widely, as might be expected.[76]

Although the bacterial carbohydrates described above are not antigenic, Perlzweig and Steffen [77] prepared a pneumococcus antigen which immunizes mice to multiple lethal doses of pneumococci, and which contains only a trace of nitrogen, resists tryptic digestion, is soluble in 90 per cent alcohol but not in ether or absolute alcohol, and is of itself not toxic for mice. Schiemann and Caspar [78] also secured more active protection of mice by immunizing with the "protein-free" precipitable specific substance of pneumococci than with the pneumococcus protein itself. Too little is known about these antigens as yet to permit us to be sure that they are actually protein-free, and still less to make assumptions as to their exact nature.

There is no theoretical reason apparent why complex carbohydrates should not exhibit antigenic functions. They may exist in colloidal state, and, as Pryde [79] has emphasized, it is possible to secure an enormous variety of carbohydrates. Even as the score of known amino acids of proteins may be built up into an incredibly great number of combinations, sugar molecules may be linked together in a vast number of ways. Two molecules of *d*-glucose may combine in at least twenty-eight ways, and Pryde calculates that 12 molecules of glucose could form somewhere in the order of 10^{18} possible different carbohydrates. However, as yet there has been adduced little evidence that carbohydrates do serve as antigens. Possibly the preparations so far tested have been denatured in separating them from the bacteria.

Nucleoproteins

If a protein radical is non-antigenic the addition of other non-antigenic radicals does not produce an antigenic compound, at least as far as now known. This seems to be true for the important group of alpha nucleoproteins, which consist of compounds of nucleic acid with protamines or histones.[80] Under the general term "nucleoprotein" has been included a variety of indefinite compounds. Inspection of the

[75a] Heidelberger and Goebel, *J. Biol. Chem.*, 74:613 (1927).
[76] For detailed consideration see Heidelberger, *Physiol. Rev.*, 7:107 (1927); *Chem. Rev.*, 3:403 (1927).
[77] *J. Exp. Med.*, 38:163 (1923).
[78] *Z. Hyg. Infekt.*, 108:220 (1927).
[79] "Recent Advances in Biochemistry," Blakiston, 2nd ed., p. 344. 1928.
[80] Wells, *Z. Immunität.*, 19;599 (1913).

literature shows that the material usually has been obtained according to Woolridge's method for isolating nucleoproteins, or some modification thereof. This method is delightfully simple, at least in principle. It consists essentially in an extraction of finely divided tissues with distilled water, filtration, and then acidifying slightly, usually with acetic acid; a flocculent precipitate is now obtained which is labelled "nucleoprotein," used as such, and the results interpreted accordingly. Commonly, to facilitate extraction, the solvent is made slightly alkaline, and for obtaining the "nucleoproteins" of bacteria, investigators have often used solutions made strongly enough alkaline to disintegrate the bacterial membranes, i.e., 1 per cent KOH, or even stronger solutions. If further refinement of the material is desired, which has not seemed necessary to many invstigators, this is accomplished by redissolving the precipitate with weak alkali and reprecipitating with acetic acid, as often as thought necessary.

The assumption that this precipitate represents pure nucleoprotein may well be questioned. Cells extracted with an alkaline solution would certainly yield a multitude of substances, many of which would be precipitated with acids. Not only would nucleoproteins and nucleins be present in such a solution, but also mucin (which is said to be universally present as an intercellular cement), "nucleoalbumins," probably various glycoproteins besides the mucins, simple globulins and albumins, and alkaline proteinate formed by the action of the alkali upon the native proteins. With slight acidification all these, with the exception of simple albumin, might be precipitated more or less completely, and such a mixture, together, probably, with many other undetermined cell constituents, would constitute the material which many investigators have called "nucleoproteins."

As for extractions made with distilled water or physiological salt solution, these can contain only such nucleoproteins as are bound to bases, for the free nucleoproteins are insoluble in water or weak salt solutions; and, for the same reason, such an extract will consist of much the same material as the alkaline extracts but in lower concentration. Even if by repeated solution and reprecipitation a purification of the material is sought, it is extremely doubtful if anything like a pure nucleoprotein will be obtained. In the first place, the various substances enumerated above will behave much the same as the nucleoproteins and accompany them in greater or less amount as long as the purification is continued. Secondly, the action of the acid and alkali will undoubtedly greatly alter the character of the original nucleoprotein,

chiefly by denaturizing the proteins so that they become insoluble, leaving the nucleins and nucleic acid in increasingly large proportions.

But most important of all is the question of the nature of the nucleoproteins themselves. The substances which have been isolated and designated under this title are, undoubtedly, salts of protein and nucleic acids. As pointed out by Osborne and Harris,[81] the nucleic acids are multibasic, e.g., salmon and wheat nucleic acid are 6-basic, so that they can unite with from one to six molecules of protein, which might be all either the same or different. Furthermore, as all protein molecules have the ability to unite with several acid radicals, the possible complexity is increased. It seems probable, therefore, that in the living cell the nucleic acids must exist bound to protein molecules, but it is highly doubtful that these compounds are the same as those which are precipitated from either neutral or alkaline extracts of the cells or tissues, a fact universally recognized by physiological chemists. With an abundance of proteins of many sorts and conditions present in such extracts and in view of the easy dissociation of the compounds of nucleic acid and proteins, it is to be supposed that the nature and proportion of the protein which is thrown down with the nucleic acid will depend entirely upon the conditions existing at the time. Variations in the concentration and character of the proteins, in the proportion of nucleic acid, of the concentration of salts and other solutes, of the degree of acidity or alkalinity of the solution, and perhaps even of the temperature, will all serve to cause variations in the composition of the precipitate which contains the nucleic acid. If the precipitation is repeated, more and more of the protein becomes insoluble in the form of albuminates, while the resulting material (as shown by Bang) [82] becomes richer and richer in phosphorus, until it becomes of the same character as the "nucleins" which are formed by peptic digestion of nucleoproteins or of tissues containing them.

Neither is there any evidence that the nucleoproteins have for their protein elements any special and characteristic sort of proteins. Osborne and Harris state that nucleic acids may combine with simple albumin and globulins, and that the resulting compounds behave as do the corresponding proteins when combined with any acids.

From these considerations of the nature of nucleoproteins it seems evident that we have to deal with three sorts of substances, as regards immunity reactions. One, the nucleic acid itself, which is non-protein,

[81] *Z. physiol. Chem.*, 36:122 (1902).
[82] *Beitr. chem. Physiol. Path.*, 4:115 (124).

practically a glucoside in fact; the nucleins, which are compounds of doubtful character, but which seem to consist of nucleic acid bound firmly to proteins, especially to the most basic of the proteins, the histones, and sometimes, perhaps, to protamines; third, the nucleoproteins, which would seem to be very indefinite and loose compounds of any or all the proteins of the cell with either nucleic acid itself, or with the nucleins.

Obviously, if the isolated nucleoproteins as such are considered, we are dealing with artificial substances which are of most uncertain and doubtful character, probably never alike in any two different preparations, and owing their antigenic character chiefly if not entirely to the abundant and loosely bound proteins. That any particular protein is specifically combined with nucleic acid to form nucleoproteins there is no evidence whatever, but, on the contrary, there is evidence that many sorts of proteins may be thus united; undoubtedly this is the case in the living cell.

Perhaps the nearest thing we have to definite protein-nucleic acid compounds are the protamine and histone nucleinates. On account of the high content of diamino-acids in protamines and histones they are strongly basic, and therefore should be particularly firmly bound by such a polyatomic acid as nucleic acid, and some at least of the nucleins are of this nature. Therefore, the question arises—can there exist a compound of nucleic acid and a histone or a protamine which possesses specific antigenic properties, characteristic of the cell from which it is derived?

Nucleic acid would not be expected to act as antigen, since it contains no protein. I have prepared sodium nucleinate from the sperm of the cod, and found that guinea pigs injected with this material were not sensitized to the same nucleinate nor yet to the albumin of the cod sperm.[83] A. E. Taylor was unable to obtain any evidence of the formation of a cytolytic immune body by injecting rabbits with nucleic acid prepared from the sperm of salmon.[84] Abderhalden and Kashiwado[85] also failed to secure anaphylactic reactions with nucleic acid from thymus nucleoproteins.

As to the histones, I have found that the "Gadus histone" of Kossel and Kutscher, prepared from cod sperm, is of itself highly toxic, and that its toxicity is not decreased by heating at 56° for 30 minutes; however, guinea pigs previously injected with this histone showed no

[83] *J. Infect. Dis.*, 9:166 (1911).
[84] *J. Biol. Chem.*, 5:311 (1908).
[85] *Z. physiol. Chem.*, 81:285 (1912).

increased sensitivity to a second injection. Likewise, Taylor [84] found no evidence of the formation of a cytolytic antibody in rabbits immunized with the protamine of salmon sperm, although immunization with entire sperm produces an antibody.

Schittenhelm and Weichardt [86] have called attention to the fact that a toxic histone becomes non-toxic when united with nucleic acid to form nucleohistone, as is also the case when toxic globin is combined in the form of hemoglobin. Gay and Robertson,[87] who found that the protamine salmin is highly toxic and that this toxicity is removed by union of the salmin with casein, also found that salmin has no antigenic power as indicated by the complement fixation test, and that it did not alter the specificity or antigenic power of casein to which it is bound. Likewise they found globin to be non-antigenic,[88] both as regards anaphylaxis and complement fixation, and globin caseinate showed no properties other than those of casein, except that globin-caseinate antiserum gives a fixation reaction with globin.[89]

If, then, histones, protamines and nucleic acid are not antigenic, it is not surprising that nucleins composed of these radicals will not be antigenic, and, according to the best evidence that I can obtain, this is the case. Preparations after Woolridge's method, but reprecipitated several times, consist practically of nucleins, the albumin being denaturized and removed in insoluble form by the manipulation involved in purification. Such preparations have been repeatedly tested in my laboratory, and found to be incapable of causing anaphylactic reactions in guinea pigs, but if the original first precipitates, which are rich in proteins, are used, strong reactions will be obtained. Even the most carefully purified preparations will, however, in large doses sensitize to the serum of the animal from which the nucleoprotein is derived, indicating that a certain amount of serum protein or equivalent tissue protein is still present in our preparations.

A series of experiments by Lake [90] has shown that even when nucleoproteins or nucleins have lost their power of causing anaphylactic reactions, they may still be able to cause the development of precipitins and complement-fixing antibodies. These antibodies are not, however, specific for the nucleins or nucleoproteins, but react with isolated globulin and albumin of the same organ, as well as with the serum of the

[86] *Z. exper. Pathol. Ther.*, 11:69 (1912); *Münch. med. Woch.*, 59:67 (1912).
[87] *J. Exper. Med.*, 16:479 (1912).
[88] Improved methods of preparation now furnish globin with antigenic properties (*see* p. 45).
[89] *J. Exper. Med.*, 17:535 (1913).
[90] *J. Infect. Dis.*, 14:385 (1914).

same species, and without even any quantitative difference in favor of the homologous nucleoprotein.

From these observations it would seem that the antigenic properties of nucleoprotein preparations depend simply upon the proteins which may be present in these preparations, and which are not in any sense a characteristic integral part of a definite substance, nucleoprotein, but rather an adventitious impurity, the character and amount depending entirely upon the method used in preparation. Such a conception serves best to harmonize the highly discordant results recorded in the literature.[91]

Hemoglobin

This is a typical compound protein, containing the non-antigenic radical, hematin, combined with a strongly basic, histone-like protein, globin. The antigenic activity of hemoglobin was formerly thought to be low, and at one time was disputed, but Hektoen has found it to be really a potent antigen.[92] The antigenic capacity of globin itself was also long doubted, most of the tested preparations being probably of too slight solubility in the body fluids and too much denaturized to exhibit any activity.[93] The earlier positive results of Browning and Wilson with globin as antigen, have been confirmed by Hektoen and Schulhof[94] and by Ottensooser and Strauss.[95] Using less damaging methods for isolation than were formerly available,[96] it was found that globin may be separated in an antigenic form. Ottensooser and Strauss found it even more antigenic than hemoglobin itself, at least in the production of complement fixation and precipitin reactions, although no success was obtained in anaphylaxis experiments. Nitro- and amino-globin compounds, which are less basic and more soluble than globin, are still more active antigens. Antiglobin serum does not react with hemoglobin of the same animal species, according to Ottensooser and Strauss, and only with globin of the same species, so that hemoglobin specificity apparently depends on globin specificity.

[91] Doerr and Pick [*Biochem. Z.*, 60:257 (1914)] have ascribed antigenic activity to organ extracts, obtained by Pohl's method, which they consider to depend on a nucleo-protein, but they submit no evidence to show that their antigen is the nucleoprotein rather than other tissue proteins associated with their preparation of "nucleoprotein." Also it was not anaphylactogenic in guinea pigs.

[92] The functionally related hemocyanin is antigenic according to C. L. A. Schmidt [*J. Immunol.*, 5:258 (1920)] but Quagliariello [*Bol. soc. biol. sper.*, 1:35)] was not *successful* in his attempts to get hemocyanin antibodies. Hemocyanin is a copper protein compound of unknown composition found free in the circulating fluid of many invertebrates, where it functions as an oxygen carrier, but it is altogether different from hemoglobin in its chemical make up, the protein radical apparently being a globulin.

[93] *See* Hektoen and Schulhof, *J. Infect. Dis.*, 31:32 (1922); 33:224 (1923).

[94] *J. Infect. Dis.*, 41:476 (1927).

[95] *Biochem. Z.*, 193:426 (1928); full review.

[96] Hill and Holden, *Biochem. J.*, 20:1326 (1926).

Hektoen and Schulhof [97] obtained active precipitins on immunizing rabbits with hemoglobin purified according to recognized methods, these precipitins being specific for hemoglobin of the same species [98] and apparently distinct from the antibodies formed in response to immunization to corpuscle stroma. Conversion of the hemoglobin into carboxyhemoglobin, sulfhydrohemoglobin, or methemoglobin does not affect its specific antigenic properties. Repeated crystallization or treatment with aluminum cream for purification does not diminish the antigenic activity of the hemoglobin. The chemical specificity of hemoglobin of different species has been shown by the characteristic crystallography of different hemoglobins by Reichert and Brown, and the crystallized hemoglobin retains its antigenic activity and specificity (Heidelberger and Landsteiner).[99] Also, a study of the effect of solution of one hemoglobin on the solubility of another hemoglobin [99a] furnishes further evidence that their chemical relationships correspond to their immunological relationships.

Racemization of Proteins

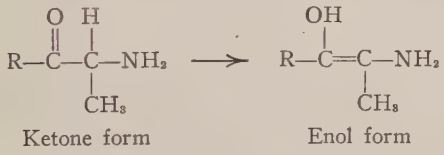
In experiments reported in 1909 it was found that treatment of crystallized egg albumin with hydrochloric acid in such a way as to form the so-called acid albumin, did not destroy its antigenic activity. On the other hand, the formation of alkali albuminate by the conventional methods completely destroyed the antigenic capacity (Wells).[100] It was later suggested by Dakin,[101] that the effect of alkalies on proteins depends on a keto-enol tautomerism of the —CH—CO— groups, resulting in a loss of optical activity and a complete loss of the capacity to undergo hydrolysis by proteolytic enzymes, or even by putrefactive bacteria. Ten Broeck,[102] using Merck's egg albumin rather than crystallized egg albumin, found that the racemized mixed proteins of his preparation were non-antigenic. Racemized zein, gliadin, casein and egg albumin, and proteoses from these racemized proteins, were likewise found to be non-antigenic by Landsteiner and Barron [103] and by Kahn and McNeil.[104]

The importance of this observation lies in its bearing on the ques-

[97] *J. Infect. Dis.*, **31**:32 (1922); **33**:224 (1923).
[98] Confirmed by Higashi, *Japanese J. Biochem.*, **2**:315 (1923); Heidelberger and Landsteiner, *J. Exp. Med.*, **38**:561 (1923).
[99] *J. Exp. Med.*, **38**:561 (1923); also Engelhardt, *Biochem. Z.*, **163**:187 (1925).
[99a] Landsteiner and Heidelberger, *J. Gen. Physiol.*, **6**:131 (1923).
[100] *J. Infect. Dis.*, **9**:147 (1911).
[101] *Jour. Biol. Chem.*, **13**:357 (1912); **15**:263 and 271 (1913).
[102] *J. Biol. Chem.*, **17**:369 (1914).
[103] *Z. Immunität.*, **26**:142 (1917).
[104] *J. Immunol.*, **3**:277 (1918).

tion of the relation of antigenic activity to proteolysis. One theory of the fundamental principle of immunity reactions is that they all represent par-enteral digestion of foreign proteins that have gained access to the tissues of the body. According to this conception, in an ideal normal condition foreign proteins never enter the body. Those taken with the food are all hydrolyzed in the alimentary canal until they have lost their original character, and are absorbed, not as foreign proteins but as simple amino acids and polypeptids which are the same as those already present in the tissues. Even the deadliest of snake venoms is harmless when taken into the stomach, for its poison is apparently a protein which cannot be absorbed intact in harmful amounts. When bacteria and other parasites pass through the epithelial surfaces of the body, or when foreign serum or other proteins are artificially injected, the situation is met as it is when foreign proteins enter the alimentary canal—they undergo proteolytic hydrolysis until their character as foreign proteins is removed. The immunological reactions, under this hypothesis, consist in an augmentation of this capacity of the body to accomplish parenteral digestion and thus to destroy harmful foreign proteins. This view receives strong support by the demonstration of the fact that proteins lose simultaneously their antigenic capacity and their digestibility when racemized by alkalies.[105] Such racemized proteins are soluble, give the typical protein reactions, retain their coagulability by heat, and apparently contain their complete quota of amino acid molecules. The only chemical and physical change seems to be the enolization of such amino acids as have their amino radicals linked to a carboxyl radical within the protein molecule, while the terminal amino acid groups containing a free carboxyl group remain unchanged (Dakin).

This is readily seen by considering the change in an alanine radical forming part of a protein or peptid molecule, as shown below.



Ketone form Enol form

The α carbon atom having by this enolization lost its asymmetry will form equal amounts of the two isomers and optical activity will be lost.

[105] This finding of Dakin and Dudley was not confirmed by Lin, Wu and Chen, *Chinese J. Physiol.*, 2:131 (1928).

When such racemized proteins are hydrolyzed some of the amino acids recovered are in an optically inactive form while others retain their optical activity, suggesting that the latter have occupied a terminal position in the polypeptid linkage, and thus escaped racemization. With the racemization come two important alterations in the properties of the protein, loss of antigenic activity and of digestibility by proteolytic enzymes.[106] Such racemized proteins injected subcutaneously are excreted unchanged in the urine (Dakin and Dudley) showing that they cannot be attacked by the enzymes within the body. Therefore, it is altogether reasonable to make the assumption that their lack of antigenic power depends on their lack of digestibility, the corollary being that antigenic activity is dependent on parenteral digestive proteolysis of foreign proteins.

The significance of digestibility as an essential factor in the antigenic action of proteins is brought into question by Landsteiner, who produced acetylated protein preparations which are effective antigens despite complete resistance to trypsin and pepsin, at least in the reagent glass.[107] However, he did not determine that the acetylated proteins also resist intestinal and parenteral digestion, as Dakin and Dudley did with their racemized proteins. Landsteiner proposes, as an alternative hypothesis, that the inert proteins merely lack the chemical structure necessary to stimulate antibody formation, independent of their digestibility, just as gelatin is digestible but non-antigenic.

Landsteiner and Barron [108] found that horse serum which had been treated with NaOH not only lost its antigenic function, but also would not bind antibodies for horse serum. If this racemized serum is treated with concentrated nitric acid and the alkali proteinates converted into xanthroproteins, this xanthoprotein is antigenic and behaves in all respects like a xanthoprotein prepared by nitration of natural horse serum which has not first been acted upon with alkali. The digestibility of this xanthoprotein was not tested. This indicates that racemization is reversible, at least as to its effect on antigenic action, but, as far as we can learn, the chemical reversibility of the racemization of proteins has not been tested. The fact that racemized proteins may be hydrolyzed by acids and yield mixtures of active and inactive amino acids indicates that the racemization is not reversible, or at least not completely reversible, and throws confusion into the explanation of the observed

[106] Racemized proteins may be hydrolyzed by acids, however, and the proteoses thus obtained may have quite the same toxicity and physiological effects as proteoses from unracemized proteins. [Underhill and Hendrix, *J. Biol. Chem.*, 22:453 (1915)].

[107] Landsteiner and Jablons, *Z. Immunität.*, 21:193 (1914); Landsteiner and Prásek, *Biochem. Z.*, 74:388 (1916).

facts. Furthermore, Kober [108] was unable to secure spectroscopic evidence of the keto-enol tautomerism suggested by Dakin as the explanation of racemization, and advances theoretical reasons indicating that the proteins cannot have as many free terminal amino and carboxyl radicals as Dakin's hypothesis requires, for this makes the assumption that all the active amino acids obtained by hydrolysis of racemized proteins must have occupied terminal positions in peptid linkages. F. C. Koch has pointed out to me, however, that Kober's criticism of Dakin's hypothesis is not conclusive, because it is by no means certain, and even doubtful, that at every peptid linkage it has gone through the enol stage.

Bacterial Toxins

In considering whether anything except intact or nearly intact protein molecules can function as antigens, we meet at once an important problem in the bacterial toxins. These substances are characterized by their antigenic capacity, and yet it is not known whether they are proteins or not. Although they behave like colloids they diffuse more rapidly than proteins usually do, and very active toxins have been prepared that do not give the ordinary reactions for proteins. This last, of course, may merely mean that the toxins are so powerful that they are active in solutions so dilute that they do not respond to these tests. As an illustration of this possibility we have the case of ricin, the poisonous antigenic toxin of the castor oil bean.

This has been isolated [109] in such purity that one-one thousandth of a milligram (0.000,001 gram) is a fatal dose per kilo of rabbit, and smaller than lethal doses lead to antibody formation, i.e., are antigenic. Nevertheless, ricin is inseparably associated with, and apparently identical with, the coagulable albumin of the castor bean. Because of the minuteness of the lethal dose some observers had thought that this vegetable toxin was not a protein, for they had obtained active solutions giving no protein reactions.

Toxins do not owe their antigenic effect solely to their toxicity, since when detoxicated with formalin or some other agents they retain their antigenic activity,[110] thus resembling proteins which also are antigenic whether toxic or not. Conversion into non-toxic "toxoids" [111] by formalin, iodin, or carbon disulfide is non-reversible but detoxification by soaps is reversible. When toxins are detoxicated with acids

[108] *J. Biol. Chem.*, **22**:433 (1915).
[109] Osborne, Mendel and Harris, *Am. J. Physiol.*, **14**:259 (1905).
[110] Ramon, *Ann. Inst. Pasteur*, 39:1 (1925).
[111] *See* review on toxoids by Bächer, *Z. Immunität.*, **54**:21 (1927).

their antigenic power is lost, returning with reactivation of the toxin by neutralizing with alkali (Doerr).[112] In this process of inactivation and reactivation with acid and alkali, toxins behave like peptids in which acids transform cyclic groups to open chains, and alkalies restore the cyclic structure.[113]

In favor of the view that toxins are proteins is the fact that they are colloidal molecules which are attacked by proteolytic enzymes. Kossel attached some significance to the fact that, like proteins, they are precipitated by nucleic acid. They are adsorbed readily by animal charcoal and similar adsorbents,[114] pass slowly through dialyzing membranes, but not at all through filters permeable only to ultramicroscopic particles of fine dimensions.[115] Apparently their molecular dimensions are smaller than those of the antibodies, and Dernby [116] has supported the view that diphtheria toxin is one of the higher degradation products of protein, presumably produced through proteolysis by the bacterial enzymes. Watson and Langstaff [117] found evidence that diphtheria toxoid, formed by action of formalin on toxin, is a proteose which contains sulfur but is free from carbohydrate.

A study of the adsorption by charcoal of different toxins showed that a bacterial hematotoxin (which dissolves red corpuscles) when adsorbed to charcoal still retained the full power to neutralize antitoxin, whereas diphtheria and tetanus toxins so adsorbed were partially inactivated (Eisler),[118] indicating that probably there are considerable differences between different classes of toxins. But the fact that toxins of many sorts are alike detoxicated by formalin without loss of antigenic activity (anatoxin formation),[119] suggests that they owe their toxicity to some common grouping distinct from whatever it is that gives them their antigenic property, or their immunological specificity. Even whole bacteria may be detoxicated by formaldehyde without affecting their antigenic properties,[120] although ricin and venoms lose much of their antigenic activity when formalinized.[121, 122]

[112] *Wien klin. Woch.*, 20:5 (1907). Also Kellenberger, *Z. Hyg. Infektionskrankh.*, 106:253 (1926).
[113] Hallauer, *Z. Hyg. Infektionskrankh.*, 105:138 (1925); 106:253 (1926).
[114] Kraus and Barbará, *Wien. klin Woch.*, 28:524 (1915).
[115] Kirschbaum, *Wien klin. Woch.*, 27:289 (1914); Glenny and Walpole, *Biochem. J.*, 9:298 (1915).
[116] *J. Gen. Physiol.*, 8:311 (1926).
[117] *Biochem. J.*, 20:763 (1926).
[118] *Biochem. Z.*, 135:416 (1923).
[119] *See Ramon, Ann. Inst. Pasteur*, 39:1 (1925); 42:959 (1928).
[120] *See* Wherry and Bowen, *J. Infect. Dis.*, 37:520 (1925); Lubinski, *Cent. Bakt.*, 104:155 (1927).
[121] Heymans. *Arch. intern. Pharmacodym.*, 32:101 (1926).
[122] For review on toxins and toxoids, *see* Loewenstein, in Abderhalden's "Handbuch der Arbeitsmethoden," Abt. XIII, pt. 2, p. 1031. 1928.

Heavy metals which precipitate proteins also precipitate toxins, which can be redissolved after removal of the metals; they come down in specific, well-defined concentrations of ammonium sulfate, and protein precipitants inactivate toxin solutions. They are usually destroyed, or at least irreversibly inactivated, by heating; slowly at 45°, quickly at 80° if in solution, but not at 100° when dried, although 150° is destructive; readily attacked by light and Roentgen rays, slowly altered by standing in solutions, not impaired by low temperatures, very susceptible to oxidizing agents and to alkalies, but much less so to acids. Also, like proteins, strong or long action of alkalies produces irreversible alterations.[123] But of course it is impossible to exclude the possibility that all these properties merely depend on the fact that the toxins are adsorbed to certain definite proteins.[124]

It is possible that toxins consist of toxic radicals attached to proteins, and that the purest toxins, which do not give chemical tests for proteins, represent the toxic radical stripped more or less completely away from the proteins with which it is usually bound. Such isolated radicals could readily reunite with the proteins of an animal into which the toxin was injected, and the resulting complex serve as antigen. Such formation of specific proteins by union of a protein with non-protein radicals is a well-known phenomenon. In this way is explained the fact that people sometimes exhibit sensitivity to non-protein substances, such as iodin, formaldehyde, salicylic acid, and other chemicals which are capable of uniting with an individual's own proteins, thus converting them into foreign proteins which serve as antigens. It is possible, therefore, that toxins are proteins to which highly toxic radicals are attached, which radicals may or may not be an essential part of the protein molecule. This possibility is supported by the fact that the venoms of different snakes of a single group (e.g., the vipers) may produce identical physiological and anatomical effects, which suggests that the poisonous element of each is the same; but the immune serum against each venom shows specific differences, indicating that the protein radicals are different for each species. Landsteiner has also shown that proteins may be combined with various organic radicals, so that the compound proteins act as antigens which produce antibodies reacting

[123] Their isoelectric point does not seem to be a very sharp one, being between pH 2.8 to 6 for diphtheria toxin, according to Predtechenskiy and Shevyakova [*Mikrobiol. J.*, 3:109 (1926)]. As determined in bouillon culture fluids it probably is not that of the toxin itself, but of a mixture or combination of toxin, bacterial proteins and bouillon proteins [Raewsky, *Biochem. Z.*, 197:8 (1928)].

[124] Bleyer [*Z. Hyg.*, 107:702 (1927)] finds that tetanus toxin and ricin are detoxicated and also lose their antigenic function when treated with certain resorcin compounds, which do not destroy the capacity of proteins to produce anaphylaxis reactions.

with any sort of protein to which the same or similar chemical groups are attached. Perhaps supporting these ideas is the fact that Nelson [125] found that the highly potent toxin of botulism is closely associated with the bacterial globulin, but remains potent, and still able to unite with antitoxin, after the globulin has been digested away by pepsin.

The potency of bacterial toxins is remarkable. Koulikoff and Smirnoff [126] report the separation of diphtheria toxin in a purified form of which the lethal dose for a guinea pig was 0.000,000,28 gm., and we know that injection of a few doses smaller than this will lead to antibody production. In view of these quantities, it is not an easy matter to isolate or study the chemical properties of toxins. Locke and Main [127] have isolated toxin from toxin-antitoxin precipitates in a purity such that the Lf unit contained but about 0.000,6 mg. N. This toxin seemed to be a lipoid-protein complex, contained no carbohydrate, was extremely sensitive to denaturation, and in its general properties and make up seemed not dissimilar to protoplasm. These authors call attention to the fact that the properties that characterize a toxin, namely, antigenicity, antitoxin-binding power, and toxicity, may well be manifestations of a single property, i.e., the combining avidity.[128]

Resemblance of Toxins to Enzymes

The facts known concerning the properties of toxins serve to throw them into the same class as the enzymes, and Oppenheimer says of the toxins, "we must be contented to assume that they are large molecular complexes, probably related to the proteins, corresponding to them in certain properties, but standing even nearer to the equally mysterious enzymes with whose properties they show the most extended analogies both in their reactions and in their activities." These similarities between toxins and enzymes are very striking. First of all we meet the same difficulty in isolating toxins that we do in isolating enzymes. A pure toxin is as unknown as a pure enzyme. At first both were believed to be proteins; now both are considered by many not to be proteins, but molecular complexes of nearly equally great dimensions. That toxins, like enzymes, are colloids, has been abundantly demonstrated. Both pass through porcelain filters, but both lose much of their strength in the process, and they are almost entirely held back by all but the most permeable dialyzing membranes. They behave similarly as regards ad-

[125] *J. Infect. Dis.*, 41:9 (1927).
[126] *Ann. Inst. Pasteur*, 41:1166 (1927).
[127] *J. Infect. Dis.*, 43:41 (1928).
[128] *See* Locke and Main, *J. Infect. Dis.*, 39:484 (1926).

sorption by suspensions,[129] and have similar effects on the physical properties of their solutions (Zunz).[130] Neither will withstand boiling, and most forms are destroyed at 80° instantly or in a very short time; on the whole, however, toxins are more susceptible to heat, as well as to most other injurious agencies. Both stand dry heat over 100°, and extremely low temperature, without much injury. Left standing in solution for some time they gradually lose their specific properties, and in each case this seems to be due to an alteration in the portion of the molecule that produces the destructive effects (*toxophore* or *zymophore* group in the Ehrlich nomenclature), while the portion of the molecule that unites with the substance that is to be attacked (*haptophore* group) remains uninjured, the toxin becoming a *toxoid,* the enzyme a *fermentoid.* On the other hand, enzymes and toxins seem to produce their effects according to different laws:—A small amount of enzyme can in course of time produce an almost indefinite amount of effect, whereas toxins act more nearly quantitatively. It seems as if the enzyme were bound to the body upon which it acts, as is the toxin, but that after it has destroyed this body it is set free in a still active form, ready to accomplish further work, whereas the toxin is either not set free, or it becomes inactive after it has once been combined.

This similarity of toxins and enzymes brings up the question of

The Antigenic Capacity of Enzymes

Although there is a considerable literature reporting positive results [131] as well as some negative results,[132] a critical review permits at the present time of no conclusion as to whether, in response to injection of fluids exhibiting proteolytic activity, there are formed specific antibodies capable of inhibiting the action of the proteolytic enzymes. The reported work is inconclusive because: (1) The existence of inhibiting substances in varying amounts in the normal serum has not always been properly considered; (2) inadequate quantitative methods have been used for studying ferment action; (3) no proper

[129] By flocculation of the colloids bearing adsorbed toxins it may be possible to secure them in comparatively pure condition [London, *Compt. rend. Soc. Biol.*, **80**:756 (1917)].

[130] *Arch. Fisiol.*, **7**:137 (1909).

[131] Bertiau, *Centr. Bakt.*, **74**:374 (1914). Stenitzer, *Biochem. Z.* **9**:382 (1908) Achalme, *Ann. Inst. Pasteur,* **15**:737 (1901). Bergmann and Guelke, *Münch. med. Woch.,* **57**:1673 (1910). Joseph and Pringsheim, *Mitt. Grenz. Med. Chir.,* **26**:290 (1913). Von Eisler, *Sitzungser. Wiener Akad. Wissensch.* (Abt. 3) **114**:119 (1905). Jochmann and Kantorowicz, *Münch. med. Woch.,* **55**:728 (1908); *Z. klin. Med.,* **66**:153 (1908). Levene and Stookey, *J. Med. Res.,* **10**:217 (1903). Kirchheim and Reinecke, *Arch. exp. Path. Pharm.,* **77**:412 (1914). Halpern, *Z. Immunität.,* **11**:609 (1911). Wago, *J. Immunol.,* **4**:19 (1919).

[132] Bergell and Schütz, *Z. Hyg.,* **50**:305 (1905). Landsteiner, *Centr. Bakt.,* **27**:357 (1900). Young, *Biochem. J.,* **12**:499 (1918). Pozerski, *Ann. Inst. Pasteur,* **23**:205 (1909). Hamburger, *J. Exp. Med.,* **14**:535 (1911). Necheles, Ling and Fernando, *Am. J. Physiol.,* **79**:1 (1926).

controls have been made by immunizing with other tissue extracts not containing the enzyme under consideration; (4) the antisera usually give precipitin reactions with the proteins of the enzyme solution, which may readily adsorb the enzymes independent of any specific anti-enzyme action; (5) in some cases the antiserum inhibits the enzyme action by altering or buffering the reaction of the solution; (6) at best, the supposed positive results have generally concerned such slight quantitative effects on the enzymes that they may readily be due to unconsidered factors; (7) several experimenters have been unable to corroborate the reputed positive results.

Similar contradictory or defective evidence is presented in respect to the production of specific antienzymes [133] for rennin [134] and such non-proteolytic ferments as lipase,[135] emulsin,[136] urease,[137] catalase,[138] fibrin ferment, amylase, invertin,[139] tyrosinase and laccase or phenolase.[140] The investigation of the antigenic activity of several of these enzymes by Abderhalden and Wertheimer [141] gave entirely negative results. Such positive results as are reported may well depend on the occurrence of precipitin reactions against the proteins of the enzyme solutions, which throw down the enzymes along with the precipitates.[142]

It is evident, therefore, that despite the other points of resemblance, the enzymes are quite different from the toxins in respect to their antigenic capacity, since this is certainly very slight if not altogether lacking in all the preparations of enzymes so far investigated.

Tuberculin [143]

This presents much the same difficulties in respect to its nature and antigenic properties as the toxins and enzymes. It has no high toxicity of itself, and does not engender antitoxins, hence it cannot be classed among the toxins. Only in the animal infected with tuberculosis does it exhibit marked local or systemic toxicity, and hence it resembles the protein antigens in the anaphylaxis reactions. According to Zinsser,[144] there are two fundamental types of sensitization to the products of the

[133] Early literature given by Schütze, *Deut. med. Woch.*, 30:308 (1904).
[134] Morgenroth, *Centr. Bakt.*, 26:349 (1899); 27:721 (1900); Hedin, *Z. physiol. Chem.*, 77:229 (1912); Thaysen, *Biochem. J.*, 9:110 (1915).
[135] Bertarelli, *Centr. Bakt.*, 40:231 (1905).
[136] Bayliss, *J. Physiol.*, 43:455 (1912).
[137] Jacoby, *Biochem. Z.*, 74:97 (1916).
[138] Burnett and Schmidt, *J. Immunol.*, 6:255 (1921).
[139] Schütze and Bergell, *Z. klin. Med.*, 61:366 (1907).
[140] Bach and Engelhardt, *Biochem. Z.*, 135:39 (1923).
[141] *Fermentforschung*, 6:286 (1922).
[142] *See* Bach *et al.*, *Biochem. Z.*, 160:261 (1925); Samysslow, *ibid.*, 182:72 (1927).
[143] Full reviews by Long in "Chemistry of Tuberculosis," Wells. DeWitt and Long, Baltimore, Williams and Wilkins, 1923, p. 70; and in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, Chap. LXXVI.
[144] *J. Exper. Med.*, 34:495 (1921).

tubercle bacillus. One is the ordinary anaphylactic reaction to the proteins of the bacillus present in most preparations of tuberculin. The other is the typical skin reaction given by preparations of tuberculin even when freed from the proteins that are coagulated by heating, which process, however, leaves in solution most of the active agent which the studies of Long and Seibert [145, 146] seem to show conclusively is a true protein. It is precipitated under the same conditions as proteins, and loses its specific activity as the protein is digested with pepsin and trypsin. The proteoses present in tuberculin or produced from it by digestion are not active. In particular, Seibert [147] has found that active tuberculin can be obtained in the form of a crystallizable protein. As this protein does not lose but gains activity by repeated crystallization, this seems to prove that the active principle of tuberculin is a protein, resembling the albumins in many respects.[148] In pure form tuberculin acts as a true protein antigen, producing specific antibodies on immunizing, but, unlike the toxins, no antibody can be demonstrated that prevents its local or systemic toxic effects in tuberculous animals.[149] Like proteins it produces anaphylaxis reactions in guinea pigs sensitized with itself, and precipitins for the proteins of the tuberculin may be generated (Seibert).[150] McJunkin, Zinsser and others have suggested that the peculiar property of tuberculin to become toxic only in tuberculous animals, depends on a specific property of tuberculous tissues to split off a toxic fraction from tuberculin. Using normal but sensitized tissue (testicle of tuberculous guinea pig) Long [151] could not substantiate this hypothesis.

Mushroom Poisons

Another possible example of a non-protein antigen has been furnished by Ford,[152] who found that rabbits can be immunized to extracts of *Amanita phalloides,* and that 1 cc. of the serum of such rabbits will neutralize five to eight times the lethal dose for guinea pigs and is antihemolytic for the hemolysin of Amanita when diluted to 1-1000. As he and Abel [153] had found this hemolytic poison of Amanita to be a

[145] *J. Am. Med. Assoc.,* **85**:650 (1925).
[146] *Am. Review Tuberc.,* **13**:408, 431 (1926).
[147] *Am. Review Tuberc.,* **17**:402 (1928).
[148] Bloch and his associates [*Arch. Dermatol. Syph.,* **148**:413 (1925)] have studied *tricophytin,* which resembles tuberculin in producing skin reactions only in infected subjects. They have isolated an active material which seems to be a polysaccharide containing glucosamine. As it is readily dialyzable it seems doubtful that it is enough of a colloid to be antigenic, but this was not tested.
[149] *See* Adler, *Wien. Arch. inn. Med.,* **7**:27 (1923).
[150] *Trans. Natl. Tuberc. Assoc.,* **23**:245 (1927).
[151] *Trans. Natl. Tuberc. Assoc.,* **22**:270 (1926).
[152] *J. Infect. Dis.,* **3**:191 (1906); **4**:541 (1907).
[153] *J. Biol. Chem.,* **2**:273 (1907).

glucoside, this observation is to be interpreted as a successful production of an antibody for a non-protein poison, a glucoside. This work was further supported by successfully immunizing rabbits to extracts of *Rhus toxicodendron,* and finding that their serum in doses of 1 cc. will protect guinea pigs from 5-6 lethal doses of the poison, which was found by Acree and Syme [154] to be a glucoside.[155] Subsequent work by the same author confirmed the main point, showing that an active hemolysin can be obtained free from demonstrable protein, and that immunization with this protein-free hemolysin will result in strongly active (1-1000) antihemolytic serum.[156] The antihemolysin unites with the hemolysin in simple multiple proportions.[157] Another, non-hemolytic poison from Amanita, which Ford designates as *Amanita toxin,* was found to contain neither protein nor glucoside, and no antitoxic serum or definite artificial immunity can be obtained for it.

These observations of Ford are of so much importance in their relation to the entire question of the nature of antigens that they should be repeated for verification. If accepted as they stand they constitute the strongest evidence yet presented as to the possibility of non-protein antigens. The newer developments in immunological research, moreover, make it seem entirely plausible that a complex glucoside, which can be hydrolyzed by enzymes, can act as an antigen. If we consider the evidence that immunity consists in the development of a special power to hydrolyze foreign colloidal substances, when these substances are of such a nature as to stimulate the cells to activity, and that Abderhalden and others have found evidence that specific enzymatic properties appear in the blood of animals injected with carbohydrates [158] and fats,[159] it seems entirely reasonable that a toxic glucoside can have antigenic properties.

Lipoids [160] as Antigens

There is a large literature on this topic [161] which reports most contradictory results, from complete denial of the possibility of antigenic

[154] *J. Biol. Chem.,* **2**:547 (1907).

[155] Von Adelung [*Arch. Int. Med.,* **11**:148 (1913)] was unable to obtain antibodies for the poisons of *Rhus diversiloba.*

[156] *J. Pharmacol.,* **2**:145 (1910).

[157] *J. Pharmacol.,* **4**:235 (1913).

[158] Immunization with starch is said by Chernokhvostov [*Zhurn. exp. biol. Med.,* **8**:260 (1928)] to produce specific complement fixation antibodies.

[159] Lebedeva [*Arkh. Biol. Naouk,* **24**:57 (1924)] reports an increased lipolytic power following immunization with sunflower seed oil.

[160] The term lipoids is here used in the broad sense including neutral fats, fatty acids, phosphatids, cholesterol, etc., and actually meaning in most immunological work the heterogeneous mixture obtained on extraction of tissues and blood with fat solvents. A valuable bibliography is given by Levene, *Physiol. Reviews,* **1**:327 (1921).

[161] Early literature given by Landsteiner, Kolle and Wassermann's Handbuch, **2**:124 (1913); Jobling, *J. Immunol.,* **1**:491 (1916).

action to the view that lipoids are of more importance as antigens than the proteins themselves. Before we can accept the idea that the lipoids are actually true antigens, capable on injection of inciting the production of antibodies which react specifically with them, we must overcome certain *a priori* objections that seem to render such a conclusion improbable if not impossible. As pointed out previously, an antigen must be a substance foreign to the body of the animal which is to furnish the antibody, and, as far as all the chemical evidence goes, with lipoids this is never the case, at least as far as lipoids from animal sources are concerned, for when the lipoids of different animal species are investigated chemically it is found that they are usually the same in all species even when these are remotely separated in their zoölogical classification. Levene [162] says "it is significant that for the present, in our laboratory at least, we have failed to discover any distinction between lipoids derived from different tissues, or different species." Hence it is not to be expected that an injected lipoid can serve as an antigen since it is not foreign to the animal into which it has been injected, nor can it exhibit specificity, as Thiele and Embleton [163] pointed out long ago. In evaluating reported positive results with lipoid antigens we must always bear in mind the fact that infinitesimal amounts of protein can function as antigen, a fact which makes it almost impossible to prove that any given lipoid "antigen" is free from enough protein to serve as antigen.[164] For example, Klopstock [165] found that the serum of rabbits immunized with supposedly pure lecithin from eggs gave precipitin reactions with egg albumin.

Despite these facts, the view that lipoids may be antigenic is strongly urged by not a few investigators. One source of confusion in the past was the failure to distinguish between antigenic function and the capacity to react with antibodies, for, as pointed out elsewhere, these two properties are not always identical. Especially in the Wassermann reaction (q.v., Chapter VIII) we see lipoid mixtures successfully used as "antigen" in the complement fixation, despite the fact that such lipoidal "antigens" do not incite antibody formation when injected into animals (Fitzgerald and Leathes).[166] To be sure, numerous observers have found that tissue extracts made with fat solvents, do have more or less capacity to incite antibody formation. For example, Bang and Forssman immunized with ethereal extracts of red corpuscles and

[162] *J. Am. Chem. Soc.*, **39**:828 (1917).
[163] *Z. Immunität.*, **16**:160 (1913).
[164] *See* Locke and Hirsch, *J. Infect. Dis.*, **37**:449 (1925).
[165] F. Klopstock, *Centr. Bakt. Orig.*, **104**:435 (1927).
[166] Univ. of Calif. Publications, *Pathol.*, **2**:39 (1912).

obtained hemolysins, so they concluded that the antigen constituent of the corpuscles is a lipoid, probably a phosphatid. This work has caused much controversy and many workers [167] failed to confirm their results.[168] It is a striking fact that when purified phosphatids, from sources favorable for obtaining pure materials, are used, the results are usually negative, while the positive results are generally reported with lipoids of more or less dubious purity, and the frank admixtures of crude lipoid extracts with foreign sera give much the most active antisera.[169]

Bacterial Lipoids

"Nastin," the lipoid material from a streptothrix, has been used for immunizing by Much and others, who state that sera are obtained which give complement fixation reactions with nastin used as the antigen.[170] Similar results are described for the fatty materials from tubercle bacilli ("tuberculonastin").[171] Despite the reported positive results, the antigenic power of bacterial fats is by no means established. Borčić [172] has reviewed the literature and repeated some of the experiments of others, but failed to find that the lipoids of typhoid and diphtheria bacilli, cholera spirilla and staphylococci, freed from proteins by drastic measures, are capable of inciting agglutinin formation. Identical negative results were obtained by Beumer [173] with the lipoids from tubercle bacilli and yeast, and by Schmidt [174] with typhoid bacilli. These experiments give strong support to the opinion that the supposed positive results with bacterial fats depend upon the presence of antigenic proteins in the lipoid preparations, and not on the lipoids themselves, especially as we know that the merest traces of antigenic substances may suffice to cause active antibody production.[175] Alcohol-soluble proteins

[167] Kimura, *Z. Immunität.*, **56**:330 (1928).
[168] Review of literature by Landsteiner, *Jahresb. Immunitätsforsch.*, **6**:209 (1910).
[169] *See* Landsteiner and van der Scheer, *J. Exp. Med.*, **41**:427 (1925).
[170] Literature in *Beitr. Klinik Tuberk.*, **20**:343 (1911).
[171] Warden [*J. Infect. Dis.*, **22**:133 (1918); **23**:504; **24**:285 (1919); *J. Bact.*, **6**:103 (1921)] reported securing positive precipitin and fixation reactions, not only with fatty complexes from bacteria and red cells, but also with artificial mixtures of soaps made up to resemble the cellular lipins. Furthermore, he claimed to have produced specific antibodies by immunizing with these artificial fatty mixtures. Indeed, he stated that the fat antigens are more specific than proteins, and inferred that the specificity of antibodies is in part or wholly due to the fats of the cells. He maintained that the phosphatids and cholesterol have no part in the process, but that only true fats and the salts of fatty acids are concerned, these acting as antigens when in a proper state of emulsification. Even diphtheria toxin was looked upon by him as but a colloidal suspension of fats, and a simple suspension of 83.3 per cent oleic acid and 16.7 per cent palmitic acid with cholesterol was described as equivalent to the antigen of diphtheria bacilli. These statements, so heterodox as to seem almost fantastic, seem to have incited few published attempts to confirm them, but Dernby and Walbum [*Biochem. Z.*, **138**:505 (1923)] repeated the experiments with diphtheria toxin, and entirely failed to corroborate them in respect to the lipoid nature of diphtheria toxin or the toxin character of synthetic lipoid mixtures.
[172] *Biochem. Z.*, **106**:212 (1920).
[173] *Biochem. Z.*, **121**:127 (1921).
[174] *Z. Immunität.*, **38**:511 (1924); *See also* Kawai, *Cent. Bakt.*, **102**:423 (1927).
[175] *See* Seibert, *Am. J. Physiol.*, **71**:621 (1925).

occur abundantly in vegetable tissues, and are probably an important element in bacterial extracts, for Klopstock and Witebsky [176] found that simple alcoholic extracts of bacteria are antigenic whereas alcoholic extracts of animal tissues are not. Nevertheless, Pinner,[177] reports that alcoholic extracts of tubercle bacilli are antigens of increasing potency as the protein content is reduced by purification, a finding not hitherto obtained by others.

Lipoids as "Antigens" in Complement Fixation Reactions

Of course it is possible, and indeed probable, that bacteria contain lipoids not present in mammals, and these may possibly be sufficiently foreign to be capable of inciting antibody formation. The same reasoning might be applied to the reported antigenic activity of lipoids from lower animals. Thus, Meyer [178] reported the production of specific complement fixation antibodies by immunizing rabbits with acetone-insoluble lipoidal material obtained from tapeworms and echinococcus cysts. He found the acetone-insoluble fraction of tubercle bacilli, presumably phosphatids, to serve as antigen in complement fixation reactions with antibodies for tubercle bacilli,[179] and much more effectively than the protein residue of the bacilli, wherefore he concluded that the reactions obtained with the lipoids certainly cannot be ascribed to adherent traces of protein. Here, however, the lipoid was not exhibiting an antigenic function by producing antibodies, but merely serving as an antigen in complement fixation reactions with antibodies engendered by immunizing with tubercle bacilli. In fact, in much of the reported work in which the antigenic function of lipoids is said to be established (e.g., Meyer [180]), the observations are of this latter sort and not at all a demonstration of antigenic activity. A repetition of Meyer's work with lipoids from tapeworms, by Sachs and Klopstock,[181] failed to confirm the statement that alcoholic extracts of tapeworms have antigenic properties when used for immunizing. But Meyer [182] again reports at least positive complement fixation developed by immunizing with a cephalin fraction of tape-worm lipoids which he believes to be free from protein. Other observers have reported at least some antibody

[176] *Z. Immunität.*, **53**:170 (1927); also Przesmycki, *ibid.*, **51**:408 (1927).
[177] *Am. Review Tuberc.*, 15:714 (1927); 18:497, 502 (1928); *see also* Weigmann and Liese, *Klin. Woch.*, 7:313 (1928).
[178] *Z. Immunität.*, 7:732 (1910); 9:530 (1911); 14:355 (1912).
[179] *Z. Immunität.*, 14:359 (1912); 15:245 (1912).
[180] *Biochem. Z.*, 129:188 (1922).
[181] *Z. Immunität.*, 55:341 (1928).
[182] *Z. Immunität.*, 57:42 (1928).

formation from immunizing with alcohol extracts of tubercle bacilli,[183] and Hoeden [184] reports the production of complement-binding antisera by immunizing two guinea pigs with crude alcoholic extracts of echinococcus.

The successful use of lipoids as "antigens" in complement fixation reactions (q.v., Chapters VII and VIII) is not proof of their true antigenic nature. Here they merely produce reactions with active sera, and do not function antigenetically. MacLean,[185] indeed, found evidence that even in the Wassermann reaction the active substance is not lecithin itself, but some other unknown substance which could be obtained practically lecithin-free. Ritchie and Miller [186] could find no antigenic activity in the lipoids of serum or corpuscles. Also Kleinschmidt,[187] who accepts the antigenic nature of nastin, was unable to secure antibodies by immunizing rabbits with it. Neufeld found that rabbits immunized with lecithin developed no opsonins for lecithin emulsions.

Effect of Lipoids on Antigenic Activity of Proteins

A suggestive observation was made by Pick and Schwarz,[188] who found that the presence of lecithin increases the antigenic power of bacteria, which fact may help to explain the activity of possible traces of proteins in lipoid preparations used as antigens. This augmenting effect of lecithin on antigenic activity has been confirmed and extended in Sach's laboratory.[189] The basis for this augmentation has not been established. It may be that by emulsionizing the mixtures the surface area of the protein elements is increased, giving a greater number of contacts and hence greater speed of reaction. Lipoids readily take up proteins, and it has been found that a solution of lecithin in chloroform will take up in fine suspension such colloids as cobra venom, trypsin, rennin and even oxide of iron (Dean).[190] Therefore alcoholic extracts of tissues and crude lipoid preparations are likely to contain more or less protein. It must also be taken into consideration that in some of the experiments in which lipoids have been thought to function as antigens, the reactions of the antiserum have not been specific.[191] Fur-

[183] Dienes and Schoenheit, *Am. Rev. Tuberc.*, 8:73 (1923); Pinner, *ibid.*, 15:714 (1927); 18:497 (1928).
[184] *Münch. med. Woch.*, 71:77 (1924).
[185] Lecithin and Allied Substances, Biochemical Monographs, 1918, p. 170.
[186] *J. Path. Bact.*, 17:429 (1913).
[187] *Berl. klin. Woch.*, 47:57 (1910).
[188] *Biochem. Z.*, 15:453 (1909).
[189] *See* Klopstock, *Z. Immunität.*, 48:97 (1926).
[190] *Lancet*, Jan. 13, 1917, p. 45.
[191] Boquet and Negre, *Ann. Inst. Pasteur*, 37:787 (1923); Schachenmeier, *Biochem. Z.*, 124:165 (1921).

thermore, the serum of normal rabbits and dogs often gives positive complement fixation with lipoidal antigens, despite the lack of any previous immunization, as Kolmer and Twist [192] observed. A source of error often overlooked lies in the fact that bacteria may grow in lipoid preparations until they themselves are abundant enough to act as antigens.[193]

Although it is established that compounds or mixtures of lipoids and proteins are effective antigens, it is certainly incorrect to say, as Much [194] and others have done, that proteins owe their antigenic properties to admixed lipins, for the purest obtainable proteins, such as recrystallized egg albumin and the vegetable protein preparations of Osborne, are fully as active antigens as the unpurified proteins, and, in my experiments, crystallized egg albumin was more active than corresponding amounts of unpurified egg albumin.[195] Such observations entirely controvert the claims of Much and his school [196] that the chief function of proteins in immunization is to secure the necessary dispersion of the lipoid particles to which all the antigenic capacity is attributed by them.

Heterophile Lipoid Antibodies

Of interest in this connection are the *heterogenetic* or *heterophile* antigens [197] described by Forssman,[198] which, present in the tissues of many animals of most varied species, nevertheless incite the formation of antibodies which cause hemolysis of sheep corpuscles. This antigenic agent is soluble in alcohol, and has been re-investigated by Landsteiner and Simms (lit).[199] Landsteiner has suggested that these heterogenetic antigens consist of two parts: one, a protein, the real antigenic factor which is necessary for the production of antibodies; the other, alcohol-soluble and presumably lipoidal, has no antigenic capacity of itself but when united with the antigenic protein confers the peculiar heterogenetic specificity of this antigen. Only the lipoid part takes part in serological reactions *in vitro*. The experiments of Landsteiner and Simms supported this hypothesis, for they found that the isolated lipoidal element of the heterogenetic antigen, itself virtually non-

[192] *J. Infect. Dis.*, 18:20 (1916).
[193] Doerr and Hallauer, *Z. Immunität.*, 47:291 (1926).
[194] *Virchow's Arch.*, 246:292 (1923).
[195] Wells, H. G., *J. Infect. Dis.*, 9:147 (1911); Lewis and Wells, *ibid.*, 40:316 (1927).
[196] Full review by Hans Schmidt, "Zur Biologie der Lipoide, mit besonderer Berücksichtigung ihrer Antigenwirkung," Leipzig, Kabitzsch, 1922.
[197] *See* review by Davidson, *Arch. Path. Lab. Med.*, 4:776 (1927); Bull, "Newer Knowledge of Bacteriology and Immunity," Univ. of Chicago Press, 1928, p. 733.
[198] *Biochem. Z.*, 37:78 (1911).
[199] *J. Exp. Med.*, 38:127 (1923).

antigenic, when merely mixed with normal foreign serum constituted an efficient heterogenetic antigen.[200] But if the lipoid is injected into one ear vein of a rabbit and the foreign protein into the other ear at the same time, little or no effect is obtained, indicating that there must occur some actual union between the lipoid and the protein in order to secure any lipoid antigen effect. What the nature of the union may be that can take place so readily between lipoid and protein, is difficult to conjecture. Presumably the union is physical, and Frankel and Tamari [201] find evidence that pig serum is the most effective protein for such synthetic lipoid antigens because the proteins are less negative than in other sera and hence unite best with the negative lipoid particles.

Analysis of the effective lipoids in the organ extracts used by Landsteiner, located them in the cerebrosid or phosphatid fraction, but, unlike the cerebrosides, soluble in water or alkali.[202] Pure cerebrosides and sphingomyelin do not react to Forssmann antibodies as do active lipoid extracts. On hydrolysis the active heterogenetic antigen yields water-insoluble acids and reducing substance giving the color test for galactose.

Such substances as these lipoids, which, devoid of antigenic activity, nevertheless act specifically upon antibodies, Landsteiner has christened "haptens," and, as pointed out elsewhere (Chapter III), he and others have demonstrated the production of many other such haptens which are not lipoidal. The Sachs school does not accept Landsteiner's hapten theory, i.e., that the lipoids are bound to the proteins as a complex antigen. Their idea is that the foreign proteins serve merely to guard the lipoids from the protective mechanisms of the injected animals, thus permitting the lipoids to reach the sites where antibodies are formed, and here independent antibodies are produced against the lipoids and against the foreign proteins. It is indeed true that antisera of animals thus immunized to lipoid-protein mixtures do react independently with the lipoids and the proteins,[203] but this observation still fails to prove that the lipoids have themselves functioned as antigens. We still must face the improbability that non-foreign lipoids can stimulate antibody formation (see p. 57). We cannot get away

[200] Other observers [Scimone and Torii, *Z. Immunität.*, **38**:264 (1923)] have found that extraction of serum with ether before injecting it as an antigen leads to the production of more specific antisera than immunizing with untreated antigenic sera.

[201] *Klin. Woch.*, **6**:2473 (1927); Tamari, *Z. Immunitäts.*, **57**:85 (1928).

[202] Landsteiner and Levene, *Proc. Soc. Exp. Biol. Med.*, **23**:343 (1926). *See also J. Immunol.*, **10**:731 (1925).

[203] Doerr and Hallauer, *Z. Immunität.*, **45**:170 (1925); Weil, *ibid.*, **46**:81 (1926); Heinsheimer, *ibid.*, **48**:438 (1926); Selter, *ibid.*, **54**:113 (1927).

from the fact that *foreign proteins must be present* to lead to antigenic activity in all these experiments. Non-antigenic agents, such as peptones, or even homologous sera, will not produce antibodies when mixed with lipoids [204] and injected into animals; hence the antigenic agent obviously is not the lipoid but the protein.

In view of all the foregoing contradictions and difficulties, it seems justifiable to say at this time that the capacity of fats and lipoids to serve as true antigens, capable of inciting the production of specific antibodies when injected into animals, has not yet been established. That such non-protein substances may, when united with proteins, modify the antigenic specificity of these proteins is now well demonstrated by the work of Landsteiner, Sachs, Doerr, and their associates.[205] Sachs and Klopstock [206] even report that isolated crude cholesterol or lecithin mixed with foreign serum constitute antigens, producing antisera on immunization which serve to react specifically with either of these lipoids. Indeed, it is said that brain lipoids can be differentiated from lipoids of other tissues by similar procedures; [207] i.e., the added lipoids are not of themselves antigenic, but they modify the specificity of the antigens to which they are united.[208] They behave the same way, apparently, as the bacterial carbohydrates which confer group specificity on the proteins of the bacteria.

Levene, Landsteiner, and van der Scheer [209] were less successful in securing antibodies specific for purified lecithin isolated from eggs and brain than reported by Sachs, which fact leaves still open the question whether it is the lecithins or admixed unknowns that are responsible for the observed effects. The fact that the tissue lipoids as used in such experiments have been found to exhibit specificity [210] is of itself evidence that something besides the lipoids is responsible for their immunological activity, for the present known chemical nature of these lipoids does not permit of their exhibiting the necessary variations to account for specificity, as is possible with proteins and carbohydrates.[211]

[204] *See* Doerr and Hallauer, *Z. Immunität.*, **47**:291 (1926).

[205] *See* review by Klopstock, *Z. Immunität.*, **48**:97 (1926); Landsteiner, *Klin. Woch.*, **6**:103 (1927).

[206] *Biochem. Z.*, **159**:491 (1925).

[207] Brandt, Guth, and Müller, *Klin. Woch.*, **5**:655 (1926).

[208] Digestion of lipoids with cobra venom, which splits lecithin, impairs the activity of the lipoids [Simeons, *Z. Immunität.*, **51**:369 (1927)].

[209] *J. Exp. Med.*, **46**:197 (1927).

[210] *See* Bordet and Reneaux, *Compt. rend. Soc. Biol.*, **95**:887 (1926); Witebsky and Steinfeld, *Centr. Bakt.*, Beiheft, **104**:144 (1927).

[211] F. Klopstock [*Centr. Bakt.*, Orig., **104**:435 (1927)] has reported securing complement fixation reactions with the serum of rabbits immunized with an optically inactive synthetic cephalin, using either the cephalin or other lipoids as Wassermann antigens. Although with such a synthetic cephalin the possibility of admixture of antigenic proteins is excluded, in view of the known readiness with which the blood of rabbits exhibits positive Wassermann reactions, this observation of itself does not suffice to establish the antigenic function of lipoids. Further development of this line of investigation will be required.

If further chemical investigation should disclose the existence of hitherto unknown, specific lipoids, the grounds now existing for doubting the existence of antigenic lipoids will need reconsideration.

Recapitulation

In order that a substance may act as an antigen it must exist as a colloidal solution, it must be foreign to the animal producing the antibodies, and it must penetrate beyond the epithelial surfaces which protect the body effectively against foreign colloids. Apparently any complete foreign protein molecule soluble in the body fluids of an animal may serve as an antigen, except proteins that have undergone racemization by alkalies. Gelatin represents the largest soluble protein molecule known which is not antigenic. Such smaller amino acid complexes as the protamines and histones are not antigenic.

Coagulation of proteins removes their antigenic capacity only to the extent that it prevents their solution in the body fluids, for if the coagulation is reversible the redissolved protein possesses antigenic capacity.

Cleavage of the protein molecule practically destroys its antigenic capacity, even when all the fragments are used together for immunizing. None of the isolated fragments of protein hydrolysis exhibits any considerable antigenic capacity, and a bare suggestion of antigenic capacity is exhibited by only a very small proportion of the larger fragments. It is not known just what step in the cleavage destroys the antigenic power of the protein molecule, but presumably the protein residue is antigenic as long as the molecules are too large to diffuse readily into the cells and therefore require the development of extracellular activities (i.e., antibodies) to accomplish their destruction. Therefore, when the fragments of protein cleavage are resynthesized to form colloidal molecules (plasteins) these are antigenic.

Compound proteins, whether natural or artificially prepared, are antigenic if soluble in the body fluids. The chief antigenic compound proteins occurring in nature are nucleoproteins, glycoproteins, and hemoglobin, and in these the non-protein radicals, being of very limited variety, seem to have little influence on the reactivity or specificity of the compound protein.

Addition of various non-protein radicals to protein antigens may alter their specificity, and simple chemicals uniting with the proteins of an animal may render them foreign to this animal so that it pro-

duces antibodies to its own altered proteins. The antibodies to such artificial compound proteins may be capable of reacting specifically with the non-protein radical, although the latter alone is not a true antigen, for it cannot incite antibody formation when not combined with a protein. These facts probably explain the supposed antigenic activity of various non-protein substances, and it is still not proved that any non-protein substance can function as an antigen.

There is evidence that some toxic glucosides may serve as antigens, which is quite possible if they represent foreign colloids.

Although many reports exist which indicate that lipoidal suspensions may serve as antigens, this has not yet been satisfactorily established. The fact that, as far as now known, the lipoids are of limited number and not specific between different animals, makes it difficult to imagine that they can incite the tissues of an animal to antibody formation when they are not different from the lipoids already present in these tissues. Possibly lipoids from bacteria and lower animal forms, such as helminths, may be sufficiently foreign to the mammalian tissues to incite antibody formation, but it is not probable that the lipoids are so different in different species of bacteria or helminths as to account for the specificity of antisera obtained by immunizing with bacterial or helminth extracts. Purified lipoids are not antigenic. Presumably the crude lipoids that are used successfully for immunizing also contain admixed protein antigens, and it is established that lipoids may unite with proteins and modify the specificity of the resulting lipoprotein antigen.

There is some evidence that substances may be obtained from bacterial cultures which are antigenic despite their failure to give any of the reactions of proteins in the solutions used for immunizing, but as yet these have been too little studied to permit the conclusion that they really do represent protein-free antigens. The typical soluble bacterial toxins, which are the antigens for antitoxin formation, come into this class of substances of unknown nature, actively antigenic even when purified so much that they do not give ordinary protein tests, but nevertheless these toxins are of colloidal dimensions. They bear numerous resemblances to the enzymes but the enzymes themselves apparently have little if any specific antigenic capacity.

Although the carbohydrates often occur in colloidal state, and are capable of forming aggregates of sufficient complexity to exhibit quite

as wide a range of specificity as the proteins, as yet antigenic function has not been demonstrated for even such foreign carbohydrates as those of bacteria, despite the fact that these markedly affect antigenic specificity. But it will not be surprising if antigenic carbohydrates should be discovered.

CHAPTER III

IMMUNOLOGICAL SPECIFICITY

A property common to all biological processes, so striking in its exactness and its results in whatever way it manifests itself, specificity is especially clearly exhibited and advantageously studied in the immunological reactions. Investigations on the specificity of these reactions have added much knowledge to many and diverse problems of biology, and seem to a large measure to have explained the essential basis of biological specificity in all its forms.

We see the manifestations of specificity in so many and so varied processes that many of them are taken for granted, and others are not at first thought of as illustrations of the biological specificity which rules all the processes of life. It must obtain, from the fertilization of the ovum by the specific sort of spermatozoon which alone can enter the germ cell and stimulate division,[1] on through the processes of growth which lead to the formation of specific structures characteristic of the species in form, composition and function, on to the specific reaction to injury which leads to repair by the proper tissue elements and the specific methods and means of defense against invading enemies. The autumnal reddening of the maple leaf, the yellowing of the birch are as typical examples of chemical specificity as the secretion of a neurotoxic poison by the cobra or the production of antitoxin by a horse immunized with the poison of the diphtheria bacillus. The fact that a dog can follow the trail of his master and recognize him from all others by scent alone, shows that each of us is chemically individual.

Relation of Immunological to Biological Specificity

When first the capacity of the animal body to produce antibodies in response to infection or artificial immunization was recognized, it at once became possible to settle an important biological problem in relation to bacteria. So simple were these forms, presenting so few

[1] For a discussion of specificity of fertilization *see* J. Loeb, "The Organism as a Whole," Putnam's Sons, New York, 1916.

possible points of structural differentiation, that there was doubt as to there being specific forms of bacteria with the capacity for indefinite reproduction of pure strains, which is a characteristic of more complex forms of life. But when in 1896 Gruber and Durham found that cholera spirilla and colon bacilli are agglutinated specifically by the proper immune serum, and when in 1897 Rudolf Kraus discovered that filtrates from cultures of plague bacilli and cholera spirilla gave a precipitate when added to the serum of a rabbit immunized with the corresponding filtrates, but that filtrates from other bacteria did not cause precipitation with these antisera, a final proof of the specificity of these microscopic living forms had been furnished, which has since been reiterated by many other means.[2]

So, too, the specificity of the precipitin reaction threw light of importance on the evolutionary relationship of animals in a way that would have delighted Darwin and Huxley had they been alive to witness it. In 1899 Tschistovitch found that the serum of rabbits immunized with eel serum gave the precipitin reaction specifically with eel serum, and soon numerous studies by others had shown that such reactions could be obtained by immunizing with the serum of any sort of animal, and that the specificity exhibited was striking but of quantitative rather than of qualitative character. It was found to be a rule that with relatively slight immunization an antiserum would be obtained which would react almost solely with the same or homologous serum, but if the immunization was continued until it reached a maximum degree the range of reactivity would broaden until the highly potent immune serum would yield distinct reactions with the serum of several, perhaps of many different species. It was also observed that these non-specific reactions were usually most marked with the sera of closely related species, and most certainly absent with the sera from widely remote species, and so it became apparent that immunological specificity is something closely related to biological specificity. This principle was brought out especially by the extended studies of Nuttall [3] on precipitin tests measured quantitatively with blood from a vast number of animals. He found that, in general, immunological relationships correspond closely with the accepted zoological classifications, which of course rest on a morphological basis.[4] Of particular interest

[2] As Bordet ("Studies in Immunity," Bordet-Gay, New York, Wiley and Sons, 1909, p. 497) points out, this specific antigenic property is apparently not always dependent on something essential for life, since bacteria may lose their agglutinability when cultivated on special media. Dawson [*J. Bact.*, 4:133 (1919)] has also found changes in agglutinability in colon bacilli grown on different media.

[3] "Blood Immunity and Blood Relationship," Cambridge Univ. Press, 1904.

[4] *See* reinvestigation and confirmation by Boyden, *Biol. Bull.* 50:73 (1926).

was the evidence of relationship between man and the higher apes as brought out by the reaction between an antihuman serum and the blood of apes and monkeys. This antihuman serum gave slight or no reactions with the blood of mammals other than the primates, but gave excellent reactions with the blood of primates, as indicated by the following table:

ANTIHUMAN PRECIPITATING SERUM

	Tested Against	Precipitate
34	Specimens human blood	100% *
8	Simiidæ, 3 species of Anthropoids	100%
36	Cercopithecidæ (Common monkeys)	92%
13	Cebidæ (Capuchins and Spider Monkeys)	78%
4	Hapalidæ (Marmosets)	50%
2	Lemuridæ (Lemurs)	0

* The percentages refer to the volume of precipitate formed on standing for a given time, the amount formed by the antiserum with its specific antigen being taken as 100 per cent. Antigen dilutions correspond throughout.

When the comparison is made by determining the dilution of either antigen or antibody which will give a reaction, instead of the less accurate estimation of bulk of precipitate used by Nuttall, it is found that the common monkeys are much less closely related to man than appears in the above tables. For example, Hektoen [5] found that antihuman precipitating serum which reacted with human serum in a dilution of 1-5,000, reacted with monkey (*Macacus rhesus*) serum only in a dilution of 1-100. By studying the hemagglutinins, Landsteiner [6] has been able to show that the blood of the lower monkeys differs from that of the anthropoids much more than anthropoid blood differs from human blood; indeed, human and anthropoid blood are more closely related than blood of horse and donkey, although these two species are so closely related that they can cross-fertilize.

Another manifestation of specificity is seen in the differing capacity of a given antigen to incite the production of antibodies in different species of animals, for it is found that, at least in the case of immunization with blood, the more closely related the species being immunized and the species furnishing the antigen, the less formation of antibodies there will be. For example, it is difficult or impossible to secure antibodies if we try to immunize dogs with the blood of wolves or foxes, or horses against ass blood. Immunization of an animal with blood of animals of the same species usually leads to no formation of antibodies, or, at the most, inconstant and slight antibody formation is observed.

[5] *J. Am. Med. Assoc.*, **70**:1273 (1918).
[6] *J. Exper. Med.*, **42**:841, 853, 863 (1925).

Although the first immunological studies were made either with bacteria or with blood, it was soon found that practically any soluble protein could be used as an antigen, and that immunological specificity was not always as definitely related to biological specificity, or better said, to zoological classification, as it had at first appeared. When egg white was used as antigen the resulting antiserum was found to react not only with the egg white of the same species, but with the eggs of many other species. Quantitative studies by Welsh and Chapman [7] led them to the conclusion that the white of bird's eggs must contain an antigen common to all avian eggs, as well as antigens specific for each species of birds.[8] Likewise, with milk as antigen it has been found that there is a marked reaction between the antiserum for milk of any one species and the milk of all other species of mammals,[9] although with quantitative differences in favor of the homologous milk. Further studies indicated that the casein is responsible for the common reactions, the other milk proteins for most of the specificity.

When the antigen is obtained from plants the same features of specificity are observed, and it is found that biologically related plants —e.g., wheat and rye, show marked immunological relationship, whereas entirely dissimilar plants usually show a corresponding immunological distinction.

On the other hand, after a time, when isolated proteins were utilized for immunization in place of such complex mixtures as blood, milk or egg white, the following facts were discovered:

(1). *Even in one species of animals there may occur several different antigens readily distinguishable from one another.*

(2). *A common antigen may occur in many and quite unrelated species.*

Distinct Antigens in a Single Species

To give an example of the first condition: In the hen's egg there have been demonstrated by means of the anaphylaxis reaction five distinct antigens and these correspond to five chemically distinct proteins that have been isolated from the egg.[10] Again, although crystallized albumin from hen's eggs shows practically no immunological distinction from crystallized albumin from duck's eggs, each of the three

[7] *J. Hygiene*, **10**:177 (1910).

[8] The reactions given with antiserum for hen egg yolk by yolk proteins from the most varied species [Seng, *Z. Immunität.*, **20**:355 (1913)] suggest that yolks also have common and specific antigens.

[9] Literature reviewed by Wells and Osborne, *J. Infect. Dis.*, **29**:200 (1921).

[10] Wells, *J. Infect. Dis.*, **9**:147 (1911).

proteins separable from horse serum (euglobulin, pseudoglobulin and albumin) can be distinguished from each of the other two by their immunological behavior.[11] That is, two chemically similar proteins from different biological sources may be immunologically similar, whereas chemically distinct proteins from a single animal may be immunologically distinguishable. Another illustration is furnished by milk,[9] the casein of which is distinguishable chemically and immunologically from the other proteins of the milk and also from the proteins of the serum of the same animal, whereas the globulin of the milk seems to be chemically and immunologically identical with that of the serum. On the other hand, casein from animals of different species is chemically similar and immunologically related. A striking case is that of the botulinus toxins, for the two known strains of *Cl. botulinum* are culturally and biologically practically indistinguishable, although their toxins are as distinct immunologically as the toxins of tetanus and diphtheria bacilli.

A certain but slight distinguishable specificity may be observed between proteins from different organs of the same animal,[12] which differentiation is still sharper between the tissue proteins and serum proteins of the animal.[13] Sex cells especially seem to be distinct immunologically from the body cells.[14] Numerous instances of two separate proteins from the same plant seeds showing entirely distinct immunological specificity have been described (Wells and Osborne).[15]

Common Antigens in Unrelated Species [16]

As an illustration of this situation, in the crystalline lens of all animals there seem to be proteins which are the same for all species, since an antiserum for the lens of one animal will give good reactions with the lens protein of any species, including even that of the same species of animal furnishing the immune serum.[17]

The lens contains two proteins, chemically distinguished by Mörner,[18] who identifies them as alpha and beta crystallin, there being more alpha crystallin in the outer and more beta in the inner parts of the lens. These two chemically distinct proteins were found by Hektoen and Schulhof [19] to be immunologically distinct from each other even

[11] Dale and Hartley, *Biochem. J.*, **10**:408 (1916).
[12] Fleischer *et al.*, *J. Immunol.*, **5**:437 (1920); **6**:223 (1921).
[13] *See* Salus, *Biochem. Z.*, **60**:1 (1914).
[14] Graetz, *Z. Immunität.*, **21**:150 (1914); Hektoen, *J. Am. Med. Assoc.*, **78**:704 (1922).
[15] *J. Infect. Dis.*, **19**:183 (1916). Also *see* Wells, Lewis, and Jones, *ibid.*, **40**:326 (1927).
[16] *See* Hektoen, *J. Immunol.*, **14**:1 (1927).
[17] Uhlenhuth, *Z. Immunität.*, **4**:761 (1910); Hektoen, *J. Am. Med. Assoc.*, **77**:32 (1921); *J. Infect. Dis.*, **31**:72 (1922).
[18] *Z. physiol. Chem.*, **18**:61 (1894).
[19] *J. Infect. Dis.*, **34**:433 (1924). Confirmed by Kimura, *Acta Scholae Medic. Kioto*, **8**:309 (1926); Woods and Burky, *J. Am. Med. Assoc.*, **89**:102 (1927).

when obtained from the same lenses, but identical with the corresponding proteins of lenses from even remote species. On the other hand, precipitins for the albumin or globulin of beef serum, for example, do not give reactions with solutions of either alpha or beta crystallin from beef lens. The presence of these two proteins in the lens of various species, including certain fishes, explains the observed organ specificity of antibodies for lens proteins, since "the lens of different species always contains the same two chemically and antigenically distinct proteins."

The specific identity of lens proteins irrespective of species is ascribed by Krusius [20] to a process of denaturalization which takes place in the transformation of epithelium into the special form which constitutes the lens, analogous to the hornification of surface epithelium. Animals sensitized with whole mammalian lens will react somewhat with serum of the same species, but this sensitizing power of the lens for serum resides in the least differentiated external layers of the lens epithelium, whereas the sclerosed central part sensitizes only to lens and not to serum. According to this author, extracts of horse hoof, cow horn, and human hair show, by means of the anaphylaxis reaction, the same high degree of organ specificity and defective species specificity as is observed with lens extracts.[21]

Indeed it is altogether reasonable that lens proteins, keratin, mucin, and other proteins whose function is not metabolism, should be nonspecific. Each of these proteins has quite the same function to perform in every species, and is set off from the active tissues to perform it. There is no more reason why they should be species specific than any other functioning product of cell activity, e.g., trypsin, epinephrine, thyroxin, insulin. These all are products of cell activity with a definite function, and apparently alike in all species, just as lens proteins have been found to be. So, too, the characteristic protein of the thyroid, thyroglobulin, seems to be immunologically distinct from the blood proteins of the animal from which it is obtained, although not distinct from thyroglobulin of other mammals.[22] A similar distinction from blood and tissue proteins is often seen in proteins set aside for nourishment of the offspring (e.g., casein, ovalbumin, seed proteins) and hence not participating in the metabolism of the organism from which they

[20] *Z. Immunität.*, **5**:699 (1910).

[21] An important utilization of this well established instance of specificity for a given protein is that by Guyer [*J. Exp. Zool.*, **31**:171 (1920)], who has shown that antirabbit lens serum injected into pregnant rabbits at a proper time may produce specific congenital eye defects which are transmissible through subsequent generations.

[22] Hektoen and Schulhof, *J. Am. Med. Assoc.*, **80**:386 (1923). *J. Infect. Dis.* **40**:641 (1927). (Thyroglobulin of birds is immunologically distinct from that of mammals.)

come.[23] Fibrinogen from different mammalian species seems to have close immunological relationship,[24] perhaps because it is a special protein with the same chemical behavior in all species.[25]

Heterogenetic or Heterophile Antibodies.[26]—A striking example of the existence of identical antigenic properties in materials of biologically unrelated origins, is furnished by the sheep corpuscle hemolysin discovered by Forssman, who found that many different tissue materials, when injected into rabbits, engender in the rabbits' serum active hemolytic amboceptors for sheep corpuscles. This heterogeneous antigenic property has been demonstrated in the organs of the guinea pig, horse, cat, dog, mouse, chicken, turtle, and several species of fish, although not exhibited by organs of many closely related species (e.g., pig, ox, rabbit, goose, frog, eel, man, pigeon, rat). It is not present in the red corpuscles of these animals, but is present in the corpuscles of the sheep, whose organs do not have this property. It has also been found in dysentery and other bacilli, mouse tumors and sheep spermatozoa. The "Forssman antigen" is soluble in alcohol and ether but not in water or acetone, so that it is of lipoid character and probably belongs in the phosphatid group. The lipoids, at least as now known, being chemically simpler than the proteins, must differ little, if any, in various species, which would account for the presence of the same antigen in many species. Further, this substance probably contains, besides the lipoid element, a non-lipoid fraction (protein) with which the antigenic property is especially identified. Taniguchi[27] has shown that lipoid emulsions act as the specific antigen with Forssman antibodies and give *in vitro* precipitin and complement fixation tests, and even *in vivo* cause anaphylactic shock, an antigen-antibody reaction; but that pure lipoids never themselves generate the Forssman antibodies when used for immunizing.

Landsteiner and Simms believe that the lipoid element of the Forssman antigen confers to the antigenic protein element the heterogenetic specificity, having found that tissue lipoids, themselves not antigenic, when mixed with foreign proteins constitute true heterogenetic antigens. But a foreign protein must always be present, for the lipoids alone have no antigenic effect, although they will react *in vitro* with the heterogenetic antibodies. Sordelli obtained from the lipoid antigen a cerebro-

[23] Semen, however, seems to be species specific [Hektoen, *J. Am. Med. Assoc.*, 78:704 (1922)].
[24] Hektoen and Welker, *J. Infect. Dis.*, 40:706 (1927).
[25] Witebsky reports a lack of species specificity in extracts of brain tissue [*Centr. Bakt.*, Beiheft, 104:144 (1927)].
[26] Review and bibliography given by Davidsohn, *Arch. Path.* 4:776 (1927).
[27] *J. Path. Bact.*, 24:122, 217, 241, 256 (1921).

side which was very active, whereas the sphingomyelin was inactive. Sachs, Klopstock and Weil report the production of heterophile antibodies by immunizing with lecithin or cholesterol mixed with hog serum, but Landsteiner could not secure antibodies when he immunized with pure lecithin or cholesterol and hog serum. Landsteiner and Levene [28] have reported that the specific part of the heterogenetic antigen is not soluble in fat solvents, yields a carbohydrate on hydrolysis and does not give a biuret reaction; its activity *in vitro* is increased by brain lipoids. (Further features of the lipoids in heterogenetic antibody reactions are discussed in the preceding chapter.)

The Chemical Basis of Specificity

It is to be presumed that immunological specificity must depend in large part upon differences in the proteins, since, as indicated in the discussion of antigens, these are usually if not always, and chiefly if not exclusively, proteins. The blood and fixed tissues of all animals are made up of water, inorganic salts, carbohydrates, lipins and proteins. The water and inorganic salts are quite the same things throughout all the living tissues, and hence these cannot incite antibody formation since they are never foreign materials. Likewise there is no characteristic difference in the carbohydrates found in different mammals, at least as far as now known, although theoretically there are innumerable possible combinations of carbohydrates, and no one has been able to demonstrate antibody formation on injection of even relatively complex carbohydrates found in the animal body and in bacteria. The carbohydrates, at least the complex polysaccharides, do unquestionably exhibit specificity in immunological reactions, even although not of themselves antigenic. The bacterial carbohydrates seem to be responsible for certain features of immunological specificity (see p. 80) and it is perhaps possible that antigenic carbohydrates may be found.

Although the chromatin elements of the cell seem to be the morphological structures which carry specificity in reproduction and cell multiplication, it is a striking fact that a characteristic constituent of chromatin, the nucleic acid, is quite the same substance in all animals so far investigated, although another type is found in plants. Hence nucleic acid cannot be responsible for immunological specificity, and nucleic acid is not antigenic.

As Levene also points out, the limited variety in the conjugated

[28] *J. Immunol.*, 10:731 (1925).

sulfuric acid radical of such gluco-proteins as mucin and chondrin, eliminates this as a factor of importance for specificity in immunity. The radicals of this group that are now known differ only in the configuration of the nitrogenous sugar present in their molecules, and this difference seems to have no relation to differences in the character of the tissue from which the gluco-proteins were extracted.

When we come to the lipins, using the term to include the fats, fatty acids and so-called lipoids, we find that here the number is limited, and apparently decreasing rather than increasing with further investigation of this class of substances (Levene).[29] In any event, when the lipoids of different animal species are investigated chemically it is found that they are usually the same in all species even when these are remotely separated in their zoological classification. Levene says "it is significant that for the present, in our laboratory at least, we have failed to discover any distinction between lipoids derived from different tissues, or different species." Unless later developments in chemistry lead to the discovery of hitherto unknown, specific lipoids there is no reason to expect that any injected lipoid will serve as an antigen, since it is not foreign to the animal into which it has been injected, nor can it exhibit specificity. But, as mentioned in the previous chapter, lipoids when admixed with protein antigens may lead to the production of antibodies which react *in vitro* more or less specifically with these lipoids. In this case the lipoids function to modify the specificity of the protein antigen, just as other substances that unite with proteins may do. The mere fact that alcoholic extracts of brain, erythrocytes, and other tissues, exhibit specificity with appropriate lipoid-protein antibodies, is no proof that it is the lipoids of these extracts that are functioning, for such extracts are complex and we do not know what is in them besides lipoids. With purified lipoids, Landsteiner was not so successful in demonstrating lipoidal specificity. We cannot afford to lose sight of the fact that as far as the chemistry of the lipoids is known, they seem to occur in too few varieties to account for specificity. And also it is to be kept in mind that when lipoids combine with proteins to constitute heterogenetic antigens, *these are characterized rather by lack of specificity*. That is, the striking thing about the Forssman antigens and antibodies is that they are common through a wide range of species. Presumably this is because the same sorts of lipoids are common to many species.[30]

[29] *J. Am. Chem. Soc.*, 39:828 (1917).

[30] Theoretically it is possible that lipoids might exhibit specificity on a purely physical basis despite chemical similarity, just as Willstätter has observed that different forms of

The inadequacy of all the other known components of the body to account for the wide range of the phenomena of specificity brings out all the more strikingly the adequacy of the proteins to account for specificity. Infinitely more complex in structure than the other tissue constituents, they occur in a virtually unlimited variety, and differences of chemical composition or physical properties are usually readily demonstrated between any two sorts of proteins that may be isolated from different sources or by different methods. Take, for example, so characteristic and actively functioning a protein as hemoglobin.

The Specificity of Hemoglobin

Having the same function in all species of carrying oxygen in loose combination for the benefit of other tissues, it might be expected that hemoglobin would be found to be a single uniform material. This, however, is not the case, for according to the crystallographic investigations of Reichert and Brown [31] the hemoglobins of all species of animals show readily recognizable differences. They say that the crystals obtained from different species of a genus are characteristic of that species, but differ from those of other species of the genus in angles or axial ratio, in optical characters, and especially in those characters comprised under the general term of crystal habit, so that one species can usually be distinguished from another by its hemoglobin crystals. But these differences are not such as to preclude the crystals from all species of a genus being placed in an isomorphous series. From their observations they reach the conclusion that the hemoglobins of any species are definite substances for that species. But upon comparing the corresponding hemoglobins in different species of a genus it is generally found that they differ the one from the other to a greater or less degree, the differences being such that when complete crystallographic data are available the different species can be distinguished by these differences in their hemoglobins. As the hemoglobins crystallize in isomorphous series the differences between the angles of the crystals of the species of a genus are not, as a rule, great; but they are as great as is the case with minerals or chemical salts that belong to an isomorphous group.

The hemoglobins also manifest their specificity in their absorption spectra, which vary from species to species, and even between indi-

aluminum hydroxide may adsorb different enzymes specifically [*See* Meyer, *Z. Immunität.*, 57:42 (1928)] but up to the present time there is no good evidence for such a specific property in pure lipoids.

[31] "The Crystallography of Hemoglobins," Carnegie Institution of Washington, Publication No. 116, 1909.

viduals of the same species.[32] Denaturing the hemoglobin, i.e., converting it into hemochromogen, renders it non-specific; but if the denaturation is reversed and the hemochromogen is converted back into hemoglobin, the original specificity is restored. Likewise, denaturation of egg albumin and other proteins by heat reduces their specificity, so that group specificity only is exhibited. Therefore, as pointed out by Anson and Mirsky,[33] a protein must be in its native form to exhibit its complete specificity. These authors consider denaturization to be a depolymerization, which uncovers a non-polar group which interferes with the manifestation of specificity.

The immunological study of hemoglobins from animals of different species also indicates a distinct specificity.[34, 35] Landsteiner and Heidelberger[36] have also investigated the differentiation of hemoglobins by studying their effect on the solubility of one another, and found that the hemoglobins of horse, dog, rat and guinea pig show differences when tested by this method. But the hemoglobins of horse and donkey behaved as if isomorphous, which is interesting in view of the fact that it is difficult to differentiate these two hemoglobins by immunological tests.

The Complexity of the Proteins

Proteins being complexes of amino acids, most of the 20 or so which have been found in proteins being present in every protein, the possible number of distinct and different proteins that might exist in nature is enormous, and entirely sufficient to explain what we know of the range of immunological specificity. Abderhalden has calculated that 20 amino acids could form at least 2,432,902,008,176,640,000 different compounds, and this without including the enormously greater number that might be made by varying the proportion of the different amino acids in a single protein. Certainly this fact of itself is strong support for the hypothesis that immunological specificity, and most of the other features of biological specificity, depends in large part on the proteins. They alone are antigenic, and they could furnish the infinite variety of modifications found in the multitudes of different living creatures and in their life processes. And, moreover, we find that to guard the specificity of all living forms, elaborate provisions have been taken to see that foreign proteins do not enter the cells or

[32] Anson *et al.*, *Proc. Royal Soc.* (*London*), Series B, **97**:61 (1924-5).
[33] *J. Gen. Physiol.*, **9**:169 (1925).
[34] Hektoen and Schulhof, *J. Infect. Dis.*, **33**:224 (1923).
[35] Heidelberger and Landsteiner, *J. Exp. Med.*, **38**:561 (1923).
[36] *J. Gen. Physiol.*, **6**:131 (1923).

the circulating fluids until they have been robbed of their foreign character through hydrolysis into simple amino acid complexes that lack specificity. The perfection with which the foregoing facts fit together to lead to the conclusion that specificity depends in great measure on the proteins, adds convincing force to this conclusion.[87]

Carefully isolated proteins, as free as possible from all other substances, show sharp immunological specificity, proving that proteins can determine specificity without the coöperation of other substances. Undoubtedly in cellular antigens (e.g., red corpuscles, bacteria), specificity is also determined to greater or less degree by the other constituents of the cells which, combined with the proteins, constitute complex antigens which may incite the formation of antibodies that can react not only with the antigenic proteins but with the modifying element of the complex, whether lipoid, carbohydrate, or some other radical.

The Evolution of the Proteins

The importance of the proteins in determining biological specificity raises the question as to how the proteins came to be specific, for, on this basis, evolution resolves itself into the evolution of the protein molecule. Certainly during the vast period extending as far back as geological evidence goes, there cannot have occurred any great change in the character of the proteins. The earliest known forms of life are enough like existing forms to indicate that their chemical composition must have been essentially the same as that of their living descendants; but they are not preserved in such a condition that their immunological relationships can be studied. The oldest proteins available for such purposes are those of the mummies and the mammoths, and the tests that have been made with mummy proteins show them to give reactions with antisera made by immunizing with human blood, and to sensitize animals to human proteins, demonstrating that in the relatively brief space of two to five thousand years no marked evolutionary alteration has taken place in human proteins, and also indicating that the specific character is fixed enough to remain unimpaired during all these years of preservation. Even more striking is the fact that the blood of a Siberian mammoth, possibly 25,000 years old, gave im-

[87] Another contribution to the chemical basis of specificity has been made by Kossel [*Z. physiol. Chem.*, 88:163 (1913)], who finds certain relations in the proportions and groupings of the scanty number of amino acids that make up the protamines and histones of sperm, to be characteristic of the sperm of certain species and families. Unfortunately these simple proteins are not antigenic, so we are unable to compare their immunological specificity with their chemical composition.

munological reactions which indicated its relation to the modern elephant (Friedenthal).[38] The immunological resemblance of human and anthropoid blood, or of proteins of modern and ancestral wheats (see p. 84), carries evidence of persistence of identical protein molecules for vast periods of time.

Kennaway [39] has considered this question of the evolution of the proteins, and asks:

"Why should the proteins that have been evolved possess so great a variety of amino acids, and why is it that none of the other related amino acids except these seventeen are found in proteins or elsewhere in nature? How are we to account for the fact that proteins usually contain amino acids that contain two, three, five or six carbon atoms, whereas four carbon amino acids are not found?" In the study of morphologic evolution it is customary to turn for light to simple forms in the scale of development, since the individual in its development recapitulates the development of the species. Kennaway applies the same principle when he compares the chemical structure of proteins from mammals, protozoa, yeasts, moulds and bacteria; but it seems that the chemical structure of the unicellular organisms is not so much simpler than that of more complex forms as is their morphologic make-up. This, however, is to be expected, since the chemical structure of unicellular organisms is fully as complex as that of the individual cells of multicellular creatures, if not more so. In the yeast, the moulds and the few species of bacteria that have thus far been analyzed, virtually all the known amino acids of animal and plant proteins have been found. Apparently, then, "the simplest organisms now existing do not contain a series of amino acids any more primitive than that present in the higher organisms, except perhaps as regards the inclusion of cystine.[39a] One may suppose that the present apparently stereotyped series of utilizable amino acids represents the stable outcome of a struggle long ago among simple organisms in which those which made a less suitable choice were beaten and have passed away, leaving no trace. We cannot know the biochemistry of the first organisms which appeared on the earth; the experiments and discarded compounds of that time are lost. The selection of amino acids must have taken place at an immensely remote period, for the earliest records which we have of the forms of life on the earth do not show us organisms which have any appearance of noteworthy difference in chemical composition from those which

[38] *Deut. med Woch.*, **30**:901 (1904).
[39] *Chem. News.*, **120**:13 (1920).
[39a] More recently cystine has been found in tubercle bacilli by Sullivan (H. G. W.).

exist at the present day. The doctrine of natural selection gives the impression that evolution proceeds throughout in a very gradual manner. But at the time when the amino acids were first being produced and tested, organic evolution must have proceeded very distinctly *per saltum* as each new compound was synthesized; natural selection would then act slowly and surely upon the organisms which made one or another choice, and thus the present series of amino acids was delimited."

Nor can we learn the history of protein evolution by studying the steps by which the proteins are synthesized at the present day. All the amino acids of animal proteins are synthesized by plants and bacteria; for although some experimental work suggests the possibility of a slight capacity to synthesize amino acids or proteins by animals, yet the results indicate even more strongly that this capacity is at the best very limited and of little or no significance as a usual source of proteins. Furthermore, no single amino acid has ever been found in animals that is not present in plants. Bacteria and plants can synthesize proteins from extremely simple inorganic salts; but the intermediate steps cannot be followed, for the entire process is completed very rapidly and without demonstrable quantities of intermediates accumulating. Thus, a multiplying yeast cell growing on simple mediums produces an entire new cell from inorganic salts in a few hours, and bacteria accomplish this marvelous synthesis still more rapidly. In the time taken by a yeast cell to produce another by budding when growing on a medium such as Pasteur's, containing no nitrogen but that of ammonium tartrate, it must synthesize each one of the score of amino acids and combine them as a series of polypeptids until yeast proteins are produced, and all the while carry on many other chemical operations within the compass of a yeast cell.

The Specificity of Carbohydrates [40]

Carbohydrates are capable of exhibiting immunological specificity, although as yet no carbohydrate has been found to be antigenic. As pointed out previously (p. 74) the complex carbohydrates may occur in colloidal form, and theoretically at least, could be as complex as the proteins, although at present we have no evidence of the existence of carbohydrates of any such degree of complexity. But the existence of

[40] For reviews and bibliography, *see* Pryde, "Recent Advances in Biochemistry." Blakiston, Phila., 1928, Chapter XI; Zinsser and Mueller, "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, Chapter LII. Heidelberger, *Physiol. Reviews*, 7:107 (1927); *Chem. Reviews*, 3:403 (1927).

carbohydrates that do determine immunological specificity has been abundantly demonstrated.

As shown by Avery and Heidelberger, type specificity of the different recognized types of pneumococci depends on the carbohydrates of their capsule, although the species specificity, that is, their identity as pneumococci, depends on the bacterial proteins. It is indeed remarkable that the pneumococcus carbohydrates will give specific precipitin and complement fixation reactions with antipneumococcus serum in concentrations of less than one part in a million, although absolutely non-antigenic. These specific gums occur in colloidal state, since they do not pass through dialyzing membranes, although they do pass from the blood into the urine of pneumonia patients.

Chemical study of these pneumococcus carbohydrates has shown that there are recognizable chemical differences, corresponding to the immunological differences. The gum from type I contains sugar derivatives and 5% of nitrogen. Half of this N can be removed by HNO_2, which destroys the specificity. The gum gives none of the tests for proteins. It may be composed of an amino-sugar, like glucosamine, linked to a galacturonic acid. The gums of types II and III contain no nitrogen, and hence do not lose their specificity when treated with HNO_2. They are acid while the type I gum is amphoteric. The type II gum seems to be a polysaccharide, made up mainly of glucose, and is dextrorotatory. The type III gum differs from type II in being levorotatory, much more strongly acid, and seems to be a polymeride of a glucose-glucuronic acid.

Although these carbohydrates are not antigenic after being isolated, they do determine specific characters in the antibodies formed by immunizing with intact bacteria, thus resembling Landsteiner's haptens. Apparently the carbohydrate acts only in a definite combined condition, for dissolution of the bacterial cells by such simple means as freezing, or bile lysis, produces a solution which does not incite formation of type-specific antibodies. Immunization with dissolved pneumococci results in the formation of antibodies which precipitate the pneumococcus protein, but not the soluble specific substance. Evidently the essential linkage upon which the character of the type-specific antigen depends, is extremely labile.

Avery and Heidelberger say that the fact that this specifically reactive carbohydrate is non-antigenic when separated from the other cellular constituents and is capable of inciting antibody formation only in the form in which it is present in the intact cell, forces the con-

clusion that in the latter instance it exists not merely as free carbohydrate but also in combination with some other substance which confers upon it specific antigenic properties. Immunization with intact bacteria containing this carbohydrate complex elicits antibodies which not only agglutinate the formed cells but precipitate solutions of the carbohydrate isolated from pneumococci of the homologous type. How the specific polysaccharide is combined in the cell, whether with protein or some other constituent, is not yet clear, but it is evident that the compound thus formed is the dominant and essential antigen of the cell, and the one responsible for type specificity. It would also appear to be readily dissociated into its components when the cell is dissolved.

A remarkable confirmation of the dependence of the immunological specificity of these gums on their chemical nature was disclosed when Heidelberger, Goebel and Avery found that the carbohydrate of a strain of Friedländer's bacillus was chemically similar to that of type II pneumococcus, and then, investigating the immunological specificity, found that the Friedländer carbohydrate reacted in a dilution of one in two million with antiserum for type II pneumococcus, but not with antiserum for type I or III. However, there are some chemical differences between the Friedländer and the pneumococcus II gums, which presumably accounts for the fact that saturation experiments showed that the gums of pneumococcus II and Friedländer bacillus are not identical. That is, Type II antipneumococcus serum, absorbed with Type B Friedländer bacillus, lost its power to precipitate the Friedländer soluble substance, but still precipitated the Type II pneumococcus substance. Similarly, the Type B Friedländer antiserum, absorbed with Type II pneumococcus, precipitated the Friedländer specific substance, but not that of Type II pneumococcus. If the fact that bacteria possess mutual absorptive capacity be accepted as the criterion of their antigenic identity then the failure of the organisms in question to exhibit this property may be taken as further evidence of the lack of identity of the substances involved. Granted a chemical difference between the two specific substances, it becomes necessary to account for their marked immunological similarity. It seems reasonable to assume that both contain in a portion of the complex molecule the same or a closely similar configuration of atoms. This essential similarity in molecular grouping would then determine the immunological similarity of the two substances.[41]

[41] Goebel and Avery [*J. Exper. Med.*, 46:601 (1927)] have shown that similar polysaccharides may be isolated from Types A and C Friedländer's bacillus and that these yield aldobionic acids on hydrolysis. Goebel also found [*J. Biol. Chem.*, 74:619 (1927)] that the

Tubercle bacilli also yield an immunologically specific carbohydrate (Laidlaw and Dudley, Mueller); composed largely of pentoses, and there is no doubt that specific carbohydrates will be found in many if not all bacteria. That the tissues of mammals contain such specific colloidal carbohydrates has not yet been demonstrated, although such a thing is perfectly possible, and probably is to be expected. But up to the present time an important rôle for carbohydrate in determining biological specificity, has been established only for the bacteria and perhaps yeasts.

Immunological Specificity Is Dependent on Chemical Individuality

So remarkable are the manifestations of specificity, so exquisitely delicate are many of the immunological reactions exhibiting specificity, and so wonderfully minute are the traces of proteins that may suffice to demonstrate it, that for some time it was assumed that specificity must depend on chemical or physical differences far too slight to be detected by other than immunological methods. More extensive acquaintance with the composition of the protein molecule and the application of more refined methods to its study, have suggested that *immunological differences between proteins are usually, and as far as now known always, associated with and presumably dependent upon chemical differences which can be detected by chemical or physical methods.* The same thing is true for the carbohydrates, as shown by Heidelberger's work cited above. A number of further observations to justify this conclusion will be described in the following pages.

Evidence from Vegetable Proteins

A study of a great variety of proteins isolated from plant tissues in the purest condition possible, led Wells and Osborne [42] to the conclusion that "since chemically similar proteins from seeds of different genera react anaphylactically in animals sensitized with one another, while chemically dissimilar proteins from the same seed in many cases fail to do so, we must conclude that the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule."

Among the observations on which this conclusion is based, we may

aldobionic acid of the Type A Friedländer bacillus is apparently isomeric with that of the Type III pneumococcus and is also made up of glucose and glucuronic acid. The polysaccharide appears to be a polymeride of two aldobionic acid units to each unit of glucose.

[42] *J. Infect. Dis.*, 12:341 (1913); 19:183 (1916).

cite the following examples: Seeds of peas, vetch, lentil and horse bean contain as their principal constituent a protein known as legumin, and no chemical difference can be found in the legumin obtained from these four seeds. Anaphylaxis experiments also failed to show any recognizable immunological difference between them. Here chemically similar proteins from different species of plants are found to be immunologically similar. A particularly striking case of this sort was reported by Jones [43] and Wells in the fact that the globulin from seeds of cantaloupe and of squash are chemically, crystallographically and immunologically identical. On the other hand, one often finds that two chemically different proteins from the same seed are readily distinguished by immunological reactions. Thus, the highly soluble seed proteins designated as "proteoses" are usually quite distinct from the other proteins found in the same seeds.[44]

An interesting observation has been made by the coördination of immunological and chemical studies of the alcohol soluble proteins of cereal grains. The chemical properties, as known from such data as are contained in the literature and those obtained by Gortner and Hoffman, indicate that these proteins may be divided into a "wheat group," which includes the proteins isolated from the genus *Triticum,* and a "corn group," including those isolated from maize, teosinte, kafir and sorghum. The genetic behavior and nuclear structure of these cereals have been extensively studied by plant breeders, especially with the wheat group, and these studies seem to reveal interesting evolutionary relationships. It has been shown that einkorn, apparently the ancestor of modern wheat, is characterized by having seven chromosomes; that emmer and durum, presumably intermediate stages, have fourteen chromosomes; and wheat and spelt have twenty-one chromosomes, as if their evolution had been accomplished by polymerization. The fertility or total or partial sterility of inter species is said to be in accordance with these chromosome numbers. In the "corn group" it has been found that teosinte will hybridize with ordinary corn, indicating a genetic relationship. Immunological studies [45] gave perfect agreement with the results of the chemical investigation of Gortner and Hoffman, who found that the chemical properties of these alcohol-soluble proteins indicate the existence of a "wheat group" and a "corn group" of prolamines, the members of each group being chemically similar to each other. Our results also showed that these alcohol-soluble proteins

[43] Jones and Gersdorff, *J. Biol. Chem.,* 56:79 (1923).
[44] Wells and Osborne, *J. Infect. Dis.,* 17:259 (1915).
[45] Lewis and Wells, *J. Biol. Chem.,* 65:37 (1925).

from cereal grains fall into these two classes. Gliadin from wheat and rye and the prolamines from durum, einkorn, emmer, and spelt, of the genus *Triticum*, seem to be very closely related, apparently identical, according to all our tests. The alcohol-soluble proteins from common maize, from kafir corn, and from teosinte, were found to be closely related to one another, but not to the alcohol-soluble proteins of the *Triticum* group.

Accepting the morphological evidence that the modern wheat with its 21 chromosomes has developed from the simple seven chromosome einkorn, perhaps through a fourteen chromosome stage as illustrated by durum and emmer, we find that through these evolutionary stages one of the most abundant and characteristic proteins of the seeds has retained a constant chemical composition. This would seem to go much farther than the observation of Friedenthal that the blood of a Siberian mammoth preserved in ice perhaps 25,000 years, shows close immunological relationship to the blood of modern elephants. It is perhaps more related to the observation of Landsteiner and Miller [46] that the bloods of man, chimpanzee and orang utan contain common agglutinins for red corpuscles not present in the blood of monkeys, which agglutinins presumably have existed in the blood of the anthropoids and man since these species split off from the common stock from which they and the monkeys arose.

The proteins from seeds indeed offer a particularly favorable material for the study of specificity, because being merely storage proteins for nourishment of the embryo plant they are set aside in relatively pure form and of limited variety in the same seed. They also often offer unusual readiness of crystallization or peculiar solubilities which facilitate their separation in pure form. By investigating such purified materials rather than complex mixtures such as serum or tissue extracts, much more exact information may be obtained. For example, the alcohol-soluble protein of wheat, gliadin, shows no recognizable chemical difference from the gliadin of rye, and these two proteins react immunologically as if identical, despite their derivation from plants of different species. Hordein of barley is chemically similar to gliadin but nevertheless definitely of different composition; immunologically hordein is found to be related to but distinguishable from gliadin. Similarly, another protein of wheat, glutenin, was found related to but distinguishable from gliadin, despite the common source of the two proteins in the wheat grain. A coördinated chemical and immunological

[46] *J. Exp. Med.*, 42:853 (1925).

study [47] of the proteins of several leguminous seeds brought out the fact that each species possesses two globulins, α and β, which can be distinguished both by chemical and by immunological methods. On the other hand, globulins from different beans may show close chemical similarity, in which case immunological similarity is also manifested.

Specificity of Proteins not Dependent on Entire Protein Molecule

The above and similar results with other plant proteins led to the suggestion [42] that *the entire protein molecule is not involved in determining the specific character of the immunological reactions of proteins, but this is developed by certain groups or radicals of the protein molecule and a single protein molecule may contain two or more such groups.* It was thought probable that the intact protein molecule is involved in the immunological reaction, since apparently nothing less than an intact protein molecule can act as an antigen, but that certain groups determine the specificity. The "group reactions" which are characteristic of biological reactions between proteins derived from closely related species, which usually have been interpreted as indicating the presence in related organisms of identical as well as distinct proteins, can also be exhibited by single isolated proteins because of the presence of common and distinctive groupings or radicals in the protein molecules. These views, suggested by results obtained chiefly by the anaphylaxis reaction, have received support from investigations carried out by others with different methods and materials, for example, the proteins of blood.

Specificity of Blood Proteins

The blood plasma contains five proteins that may be isolated by chemical methods, and which exhibit distinct chemical differences from one another. These proteins are fibrinogen, albumin, euglobulin, pseudoglobulin, and seromucoid. When the fibrinogen is removed by coagulation the serum contains the other four proteins. Bauer and Engel [48] found that fibrinogen from beef plasma is immunologically distinct from the proteins of the serum, since antiserum for the fibrinogen produces little or no complement fixation or precipitation with beef serum, and conversely. Hektoen and Welker [49] have found that antibodies for fibrinogen from any one mammal will react with all the other

[47] Wells, Lewis and Jones, *J. Infect. Dis.*, **40**:326 (1927).
[48] *Biochem. Z.*, **42**:399 (1912).
[49] *J. Am. Med. Assoc.*, **85**:434 (1925); *J. Infect. Dis.*, **40**:706 (1927).

mammalian fibrinogens, but fibrinogen from birds is immunologically distinct from these. However, there are distinct quantitative differences, so that an antiserum reacts more strongly against the homologous fibrinogen than against any of the others. This lack of species specificity is in harmony with the fact that fibrinogen of one species will form fibrin when acted on by thrombin from another species. However, Downs [50] has found that tissue fibrinogen is species-specific antigenically, although not species-specific as a coagulant.

The chief serum proteins, in turn, seem to be immunologically distinct from each other. As early as 1901, Leblanc [51] found this to be true, and that these proteins are also distinguishable from hemoglobin by the precipitin reaction. Dale and Hartley [52] found that a guinea pig which has received a sensitizing dose of any one of the purified horse serum proteins (albumin, euglobulin, pseudoglobulin) is more sensitive to that one than to either of the other proteins from the same serum. In some cases, indeed, the reaction was rigidly specific. Kato [53] also separated carefully from horse serum the albumin, water-soluble globulin (pseudoglobulin) and water-insoluble globulin (euglobulin) fractions. He likewise found that while guinea pigs sensitized to one of these fractions did react to the other two, yet they reacted much more strongly to the homologous fraction.[54] All these and other observers [55] have noted that serum albumin is much less effective as an antigen than the other serum proteins, but Hektoen has found it a better antigen than is usually reported. Doerr and Berger [56] not only found it possible to distinguish between euglobulin and serum albumin, but also between two fractions of the serum albumin obtained by precipitating with ammonium sulfate at 56-66 per cent and 66 to 99 per cent. According to them, five antigens may be distinguished in plasma, namely, fibrinogen, euglobulin, pseudoglobulin, albumin C and D, to which we may add seromucoid.[56a] Each fraction is specific when tried against the corresponding protein of plasma from other animals.

To add to the number of antigens distinguishable in blood are those in the corpuscles, Hektoen and Schulhof [34] having found that hemoglobin is distinct from both the corpuscular stroma, and the serum proteins, while the globulin of the red cells is immunologically distinct

[50] *J. Infect. Dis.*, **37**:49 (1925).
[51] *La Cellule*, **18**:335 (1901).
[52] Dale and Hartley, *Biochem. J.*, **10**:408 (1916).
[53] *Mitteil. med. Fak., Univ. Tokyo*, **18**:195 (1917).
[54] *See also* Kimura, *Z. Immunität.*, **56**:330 (1928).
[55] Stern, *Arch. Hyg.*, 91:165 (1922); Ruppel, Ornstein and Lasch, *Z. Hyg.*, **97**:188 (1922).
[56] *Z. Hyg.*, **96**:191 (1922). *See also* Otto and Iwanoff, *Z. Immunitäts.*, **57**:19 (1928).
[56a] Lewis and Wells, *J. Infect. Dis.*, **40**:316 (1927).

from the globulin of the serum (Bennett and Schmidt),[57] as well as from the hemoglobin.[57a] Furthermore, the globin itself when isolated seems to be immunologically distinguishable from the hemoglobin from which it was derived.[58] In addition to the specific proteins must also be included the lipoids of the corpuscles, which also exhibit their own specificity,[59] and probably the serum lipoids also.

As Doerr and Berger [60] point out, *each of the proteins of serum exhibits two kinds of specificity,* one for the species and one within the species characterizing the particular protein itself. Hence there must be at least two different groups or factors determining specificity within a single protein molecule, as Wells and Osborne had previously suggested. Studies on bacterial antigens also have led Zinsser to a similar conclusion, namely, "it seems quite likely that the antigenic function of specific union with the antibody may be dependent upon a relatively small nucleus of the protein molecule." [61] In agreement with this is the fact that Nelson [62] has found that a globulin-like protein present in several species of the colon-typhoid group exhibits a double specificity, one distinguishing group from group, the other differentiating the types within the group. The work on lipoid antigens cited in the preceding chapter, furnishes abundant evidence that a lipoid radical can confer one sort of specificity to a protein antigen which also exhibits another sort of specificity of its own.

Chemical Differences between Serum Proteins

It has been well established that the different serum proteins are chemically different. Long ago Abderhalden [63] found that horse serum albumin contains no glycine, whereas serum globulin contains 3.5 per cent of glycine. Michaelis also found that the isoelectric point of serum albumin is much different from that of serum globulin, and of oxyhemoglobin.[64] Hartley [65] has shown that serum albumin differs from serum globulins in yielding, on hydrolysis, different proportions of cystine and diamino acids; however, he did not find corresponding differences between the two globulins, and no difference can be found

[57] *J. Immunol.*, **4**:29 (1919).
[57a] Muscle hemoglobin is immunologically distinct from blood hemoglobin of the same species. [Hektoen, Robscheit-Robbins and Whipple, *J. Infect. Dis.*, **42**:31 (1928)].
[58] Ottensooser and Strauss, *Biochem. Z.*, **193**:426 (1928).
[59] Landsteiner and van der Scheer, *J. Exper. Med.*, **41**:427 (1925).
[60] *Z. Hyg.*, **96**:258 (1922).
[61] "Infection and Resistance," **1923**, p. 110.
[62] *J. Infect. Dis.*, **40**:412 (1927).
[63] *Z. physiol. Chem.*, **37**:495 (1903); **44**:17 (1905).
[64] "Die Wasserstoffionenkonzentration," **Berlin, 1914**, p. 56.
[65] *Biochem. J.*, **8**:541 (1914)

between them by racemization (Woodman).[66] Miss Chick and others have given reasons for considering euglobulin as being a compound of pseudoglobulin with a lipoid, which may account for the immunological differences. In view of these facts the reputed transformation of serum albumin into globulin by various physical methods is obviously incorrect, and Fanconi [67] found that such "artificial globulins" show immunologically that they are not globulins but merely albumins altered in solubility.

Of much significance in connection with the fact that different proteins from the same serum can be differentiated immunologically from one another, and also from corresponding protein fractions of sera of different species, is the study of Obermayer and Willheim [68] on the formol titration figures of serum proteins. The reaction in Sörensen's serum titration method seems to depend on the presence of terminal amino groups in the protein molecule. Taking as their "amino index" the quotient obtained by dividing the total nitrogen figure of the protein with the figure for the terminal amino groups as determined by formol titration (the result indicating actually how many nitrogen atoms the protein molecule has for each terminal amino group), these authors investigated the protein fractions of serum. In mammalian serum it was found that the amino index for the euglobulin fraction averages around 21.5, the pseudoglobulin gives a similar index, while the albumin is constantly much lower, average about 12. In fowl serum the pseudoglobulin, with an average index of about 15, resembles more closely the albumin figure. It was found also that, according to the evidence of the amino index, the pseudoglobulin fraction and the albumin fraction can each be separated by ammonium sulfate precipitation into two fractions differing distinctly in their composition, the latter corresponding presumably to the two albumin fractions which Doerr and Berger differentiated by immunological methods. The fact that the protein fraction of fowl and goose serum precipitated at 25-30 per cent saturation with ammonium sulfate has an amino index of from 28.5 to 32.5, whereas the corresponding fraction in horse and beef serum has an index of about 19, shows that these proteins are truly different chemically, just as they have been shown to be immunologically.

[66] *Biochem. J.*, **15**:187 (1921).
[67] *Biochem. Z.*, **139**:321 (1923).
[68] *Biochem. Z.*, **38**:331 (1912); **50**:369 (1913).

Bence-Jones and Noel Paton Proteins

As further evidence of the dependence of immunological specificity upon chemical composition of proteins, we may add the following examples:

Occasionally another protein, known as Bence-Jones protein, is found in the urine and blood of persons suffering from extensive neoplastic invasion of the bone-marrow. This protein is quite unique in its chemical and physical properties. Hence, Massini [69] found that Bence-Jones proteins can be differentiated from human serum-proteins by complement fixation tests, and Hektoen [70] corroborated this with the precipitin reaction. Bayne-Jones and Wilson [71] obtained similar results by these methods and also in anaphylaxis experiments. A preparation of Bence-Jones protein which had been crystallized was found to be free from any of the normal serum proteins. It seems probable, to judge by their immunological behavior, that there may be more than one sort of Bence-Jones protein, and the crystalline Bence-Jones protein studied by Hektoen and Welker differed from one described by Krauss in containing less carbohydrate and loosely bound sulfur. Furthermore, another crystallizable protein, chemically distinct from Bence-Jones protein, found in urine by Noel Paton, has been found to be immunologically distinct from either Bence-Jones or serum proteins.[72] Hektoen [73] has observed a case of excretion of still another crystallizable urinary protein, and demonstrated the immunological identity of this protein from both the Bence-Jones protein and the protein originally collected by Noel Paton.[74]

Milk Proteins

A study of the chemistry and immunological behavior of milk proteins has furnished further evidence of the same purport (Wells and Osborne).[75] Cow's milk contains four chemically distinct proteins or protein fractions, namely, casein, lactalbumin, lactoglobulin and an alcohol-soluble protein. By means of the anaphylaxis test it can be shown that these four proteins are immunologically distinct. This fact furnishes another striking illustration of the dependence of immunologic specificity on chemical composition rather than biologic origin. Of these

[69] *Deut. Arch. klin. Med.*, 104:29 (1911).
[70] *J. Am. Med. Assoc.*, 76:929 (1921); Hektoen and Welker, *J. Infect. Dis.*, 34:440 (1924).
[71] *Bull. Johns Hopkins Hosp.*, 33:119 (1922).
[72] Everett, Bayne-Jones, and Wilson, *Bull. Johns Hopkins Hosp.*, 34:385 (1923).
[73] Hektoen *et al.*, *J. Am. Med. Assoc.*, 83:1154 (1924).
[74] Willheim [*Biochem. Z.*, 180:231 (1927)] finds chemical evidence also for the view that various proteins are being included in this group.
[75] *J. Infect. Dis.*, 29:200 (1921).

four proteins only one, the globulin, sensitizes to beef serum or causes reactions in animals sensitized to beef serum. This corresponds to the observation of Crowther and Raistrick that milk globulin and serum globulin are chemically indistinguishable; and of Woodman [66] that lactalbumin and serum albumin are two distinct proteins.

Biologically, casein is quite as distinct from the whey proteins and the serum proteins as it is chemically, whereas the biological reactions and chemical composition of caseins from different species of animals show close relationships. Even the delicate method of spectrophotometric comparison of different caseins indicates them to be practically identical.[76] According to Fleischer [77] an immune serum for any one casein will give reactions with casein from any other species, even of remote relationship. The whey proteins as a whole are biologically similar to the soluble serum proteins of the animals from which they are derived. *Casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or to the serum proteins of its own species; the same is true of the chemical relations.*

Although caseins from different species do not show demonstrable quantitative or qualitative chemical differences by ordinary methods, a study of the products of racemization by Dakin's method yielded to Dudley and Woodman [78] evidence of some structural differences. Caseins from sheep's and cow's milk consist of identical amino acids, apparently in identical proportions, but when racemized the sheep's casein showed all the tyrosine and most of the lysine unracemized, while both were completely racemized in cow's casein, indicating that these amino acids occupy different positions in the caseins of the two species. Dale and Hartley [79] state that caseins from different species show no clear disparity of antigenic properties, but it is possible that finer quantitative methods will disclose distinct discrepancies.

Egg Proteins

Ovomucoid, crystallized albumin and ovovitellin from hen's eggs have been found to be readily distinguishable from one another by the anaphylaxis reaction.[80] By saturation tests it was shown that in crystalline egg albumin and in the globulin-fraction of hen's egg white,

[76] Abderhalden and Rossner, *Z. physiol. Chem.*, 168:171 (1927).
[77] Fleischer, *Roussky Wratsch*, (7, pt. 2) 1638 (1908).
[78] *Biochem. J.*, 9:97 (1915).
[79] *Biochem. J.*, 10:431 (1916).
[80] Wells, *J. Infect. Dis.*, 6:506 (1909); 9:147 (1911).

there are specific and distinct antigens, as well as a common antigen which cannot be separated from them by fractional precipitation with ammonium sulfate. By using the precipitin test and new methods for separation of the proteins, Hektoen and Cole [81] found that they could detect five distinct antigens in egg white (ovoglobulin, ovomucin, crystallizable ovalbumin, non-crystallizable conalbumin, and ovomucoid), only the conalbumin seems to be immunologically identical with any of the proteins of the fowl's serum (serum albumin). Therefore, there are distinguishable in the hen's egg at least six distinct antigens which correspond to an equal number of proteins which chemists had previously distinguished. On the other hand, crystallized egg albumin of duck eggs corresponds perfectly within the limits of chemical methods with crystallized albumin of hen's eggs, and each of these proteins sensitizes to the other apparently as well as to itself (Dale and Hartley).[82] Egg yolk proteins from even such widely different species as fish and turtle may give precipitin reactions with antiserum for hen egg yolk proteins.[83] Ovomucoids from eggs of birds of different species show immunological relationship, but can be distinguished by refined methods.[83a]

A particularly interesting observation bearing on the dependence of immunological differentiation on chemical variations has been furnished by the egg proteins. Dale and Hartley had found, in preliminary experiments, that crystallized albumins from hen eggs and from duck eggs sensitized animals mutually to one another, even with the guinea pig uterus method, but by using quantitative methods and by checking up with the desensitizing test, it was later possible for Dale to detect slight immunological differences between the two.[84] The importance of this observation lies especially in the studies carried out by Dakin on the chemistry of these two albumins. When hydrolyzed, their amino acid content is found to be similar, but when the proteins are first racemized and then hydrolyzed it is found that there are distinct differences in the degree of racemization of the leucine, aspartic acid and histidine. There being reasons for believing that the amino acids which escape racemization occupy the terminal positions in the peptid chains of which the protein molecule is built, this result indicates that there are at least structural or spatial differences in these two proteins. In other words, a close relationship by immunological tests is here associated

[81] *J. Infect. Dis.*, **42**:1 (1928).
[82] *Biochem. J.* **10**:408 (1916).
[83] Emmerich, *Z. Immunität.*, 17:299 (1913).
[83a] Lewis and Wells, *J. Infect. Dis.*, **40**:316 (1927).
[84] *Biochem. J.*, **13**:248 (1919).

with chemical similarity, and a slight difference in chemical structure is found which presumably accounts for a slight immunological difference that can be detected only by the most sensitive methods.

Immunological Behavior of Artificially Modified Proteins

The conclusion that *immunological specificity of proteins depends on chemical differences of a degree usually and perhaps always capable of recognition by existing chemical methods,* which, as outlined in the preceding paragraphs, has been derived by the study of the immunological reactions of isolated proteins as they occur in nature, is corroborated by the results obtained by modifying the composition of the protein molecule by artificial means. The pioneers in this field were Obermayer and Pick,[85] who found that specificity for species, as indicated by the precipitin reaction, is altered when the aromatic radicals are acted upon in various ways. This is most readily done by introducing such radicals as iodin, NO_2 and $N = N$ into the protein molecule, where they are known to enter specifically into the benzene ring of the aromatic amino acids. They report that such altered proteins produce precipitins that are specific for the form of protein used in immunizing, but not at all specific as regards the species from which the protein is derived; e.g., iodized bovine serum produces in immunized rabbits precipitins that will react only with *iodized* proteins, but they react with iodized proteins of any source, whether from egg white, horse serum, or even iodized rabbit proteins.[86] On the other hand, such modified proteins are said to be quite specific toward themselves, i.e., precipitins for iodized proteins will not react with diazotized proteins, and conversely. Furthermore, if radicals were introduced into amino acids which do not possess the benzene ring, species specificity was not affected, although the resulting proteins exhibited specificity toward themselves.

These observations, as far as they agree, indicate that the aromatic radicals may be of importance in determining species specificity. This view received some support from the observation that gelatin, which of all proteins is poorest in aromatic amino acids, possesses no antigenic

[85] *Wien klin. Woch.,* 19:327 (1906).

[86] Freund [*Biochem. Z.,* 20:503 (1909)] obtained iodized serum and egg albumin containing 6.5-8 per cent of iodin, which produced precipitins that were not species specific but specific for iodized proteins. The proteins were, however, according to these figures, far from completely iodized, and with iodized serum, and with iodized crystallized egg albumin which was known to be completely saturated with iodin, no loss of species specificity was detected by the anaphylaxis reaction [Wells, *J. Infect. Dis.,* 5:449 (1912)]. To be sure, Schittenhelm and Ströbel [*Z. exp. Path. Ther.,* 11:102 (1912)] state that iodized serum proteins sensitize to iodized egg white, and conversely, but their work is not reported in sufficient detail to establish that sensitization by traces of the intoxicating protein may not have occurred.

capacity (Wells, Starin). But Landsteiner and van der Scheer [87] found that the substitution of H in an aromatic radical with various groups, such as NO_2, Cl, I, do not necessarily greatly modify the specificity, which raises the question whether the results of Obermayer and Pick depend on union with aromatic radicals or on other changes in the protein molecules.

Pick's Conception of Specificity

The studies of Pick and his colleagues,[88] amplified somewhat by other investigations, have led him to the following view of the chemistry of specificity: There exist two sorts of specificity in each protein molecule; one of these is easily altered by simple physical measures, e.g., heat, cold, partial coagulation, etc., without essentially changing the chemical composition of the protein. When so altered the antigenic properties of the protein are likewise altered, in that the antibody it engenders differs somewhat in the scope of its reactivity from the antibody engendered by the original unaltered protein; but the alteration does not affect the species characteristics of the antigen. Thus, a heated antigen may engender precipitins that will react with this heated antigen, but not with similar heated proteins from other species of animals, while the antibodies engendered by the same but unheated antigen will not react with the heated protein. Bordet, who looks upon immunological reactions as essentially adsorption phenomena, considers that this altered specificity of heated proteins supports the conception that adsorption affinity is the essential factor in the reactions, and that specificity depends on physical rather than chemical relations of the materials involved.[89]

The other sort of specificity is not so easily affected, according to Pick, only marked chemical alterations of the antigen modifying it, and this concerns the species characteristics of the antigen. This fundamental species specificity seems to be closely related to the aromatic radicals of the protein antigen, for it is affected by introducing into the protein molecules substances which are known to combine with the benzene ring, e.g., iodin, diazo and nitro groups. Proteins thus chemically altered will act as proteins foreign to animals of the species from which they are derived, and the antigens they develop are devoid of species specificity, although quite specific for proteins like themselves; e.g., a nitro-protein made by treating rabbit serum protein with nitric

[87] *J. Exp. Med.*, **45**:1045 (1927).
[88] E. P. Pick, Kolle and Wassermann's *Handbuch d. path. Mikroorganismen*, **1**:685 (1912).
[89] "Studies in Immunity," Bordet-Gay, 1909, p. 525.

acid, will, if injected into even the same rabbit, cause the formation of antibodies which will react with this same nitro-protein, and also with nitro-proteins derived from entirely different species or even from plants,—but reacting only with nitro-proteins. It is also possible to cause chemical modifications analogous to the physical modifications previously mentioned, which change only the scope of specificity of the antigen without altering its specificity for species. Appreciating that the number of different aromatic radicals in the protein molecule is not sufficient to account for the innumerable manifestations of specificity, Pick interprets the significance of these aromatic radicals as that of a central complex about which are the groupings which determine species specificity. It is not merely the number and proportion of amino-acid radicals in the protein molecule which determine its specificity, but, more important because presenting greater possibilities for variations, the arrangement of these radicals in the molecule.

Landsteiner's Observations

The extensive investigations of Landsteiner [90] and his colleagues, however, have led them to a different opinion. They found that species specificity may be altered in many other ways than by attacking the aromatic rings of the protein. This they accomplished by esterification with acid alcohol, acetylating with acid anhydride or acid chlorides, or methylation by means of diazomethane. The common feature of these reactions is that the alterations occur in the salt-forming groups of the protein molecules, and they evidently do not depend solely on substition of H-atoms in the aromatic radicals. This artificial, structural specificity is marked, and traverses by far the biological lines; e.g., antiserum for methylated protein of horse serum may react not only with methylated proteins from most diverse animal species, but also with methylated plant proteins.

It is also possible to produce artificial protein compounds which are antigenic when injected into the species from which the protein is derived, but which engender antibodies retaining species specificity; e.g., formaldehyde-treated rabbit serum protein when injected into rabbits produces antiserum reacting with itself, but not with formaldehyde-treated proteins from other species. In this case, then, a chemical change which has made an animal's own serum proteins antigenic for itself, has failed to alter the species specificity.[91] It is believed that formaldehyde

[90] *Biochem. Z.*, 86:343 (1918). Gives bibliography.
[91] Landsteiner and Lampl, *Z. Immunität.*, 26:133 (1917).

acts on the lysine radical of proteins, forming methylene compounds of amino acid groups, R—N=CH_2. Evidently, then, the occupation of an amido group of lysine by the methylene radical is almost devoid of any effect on specificity, and a marked chemical change can take place in a protein without noticeable effect on the structural or species specificity.

This lack of effect of formaldehyde may be contrasted with the marked effect on serological specificity which results from alkylization or acetylation of the proteins, indicating that a relatively small radical added to the large protein radical may exhibit its own characteristic influence on the specificity of the entire complex.[92] Thus it was found that rabbits immunized with acetylated horse serum[93] produce complement fixation antibodies reacting with acetylated serum from other species than horse, e.g., fowl or rabbit serum, but not reacting with normal or diazotized horse serum. Here a simple added radical has deprived the horse serum of its species specificity while giving it a new structural specificity depending on the presence of the acetyl group.

An extensive series of protein compounds was made by combining horse serum with the diazonium derivatives obtained from the following amino-compounds: aniline, *o-*, *m-*, and *p*-aminobenzenesulfonic acids, a number of methyl, chlor, brom and nitro substitution products of the above compounds, *p*-aminophenylarsenic acid, *o-*, *m-*, and *p*-aminocinnamic acid, naphthionic acid and aminoazobenzenedisulfonic acid. The 23 different immune sera obtained by immunizing with these compound proteins were tested with 33 different azo-proteins. Of these immune sera, only 6 (aniline, *p*-aminobenzenesulfonic acid, *o-*, *m-*, and *p*-aminocinnamic acid and aminoazobenzenedisulfonic acid) were entirely specific, i.e., they reacted only with the homologous antigen and vice versa. Of the *antigens,* 15 reacted only with some one of the antisera and 9 of these 15 antigens were not homologous, antisera not having been prepared for them. The other immune sera and antigens showed a broader sphere of activity.

Cross reactions were obtained only with antisera that contained chemically closely related aromatic groups. The immune serum always reacted with its homologous antigen, but some of the sera also reacted with antigens that contained aromatic side chains that were either isomeric or very closely related chemically to the aromatic side chain of the homologous antigen. The important factor seemed to be the relative location, in the aromatic nucleus, of the acid and the diazo group. Cross

[92] Methylation of casein alters its specificity [Kimura, Tawara, and Toda, *Acta Scholae Medic. Kioto*, 7:449 (1925)].

[93] Landsteiner and Jablons, *Z. Immunität.*, 21:193 (1914).

reactions were obtained when these groups were identically located, irrespective of the other constituents of the nucleus, or when the groups were, say, meta instead of ortho to each other. For example, antigens prepared from diazotized *o*-aminobenzenesulfonic acid, *p*-toluidinesulfonic acid and *p*-chlor, *o*-aminobenzenesulfonic acid and *m*-aminobenzenesulfonic acid, reacted with the diazotized *p*-toluidinesulfonic acid antiserum. However, the most pronounced reaction was obtained with the homologous antigen. In the first three antigens, the sulfonic acid group is located ortho to the diazo group. In the last antigen, the sulfonic acid group is in the meta position with respect to the diazo group. In this respect some groups, especially those of acid character, were found to have a particularly marked effect.[94]

The arsenic acid immune serum reacted only with the six arsenic acid antigens. The effect of the introduction of a substituting methyl group in general is not very considerable. The substitution of Cl and Br have less influence on the serum reactions than carbonic, sulfonic and arsenic acids. The conclusion is reached that *the specific behavior is determined by the chemical structure of relatively small portions of the large antigen molecule.* The observation that the location in the molecule of a simple organic compound of definite groups can be indicated by immune serum when this compound is united to an antigenic protein, can best be explained as depending on certain spatial correspondence of antigen and antibody, just as Emil Fischer assumed for the specific action of ferments. Apparently other factors must also play a part in determining specificity, as there are some groups of reactions that do not correspond to this theory. Thus, the arsenic acid serum was strictly specific solely for arsenic acid antigens. Here the specificity must be determined by the arsenic acid group as such. Therefore, it is concluded that besides the configuration also the chemical characteristics of certain groups come to expression in serological specificity. Landsteiner holds that it is probable that an antibody can react with various related but not identical antigens. *Specificity is the expression of a quantitatively graded affinity which reaches a maximal value with a certain combination,* designated as specific antigen and homologous antibody. Hence we have antibodies with varying range of reaction with related antigens.[95]

Probably there is an enormous number of isomers of a protein and these cannot all be distinguished by the present serological methods.

[94] Landsteiner and van der Scheer, *J. Exp. Med.*, **45**:1045 (1927).
[95] *See* Landsteiner and van der Scheer, *J. Exp. Med.*, **40**:91 (1924).

Landsteiner says that his own observations would place the species specificity on the spatial configuration of the protein and raise the question if the terminal acid groups are not of significance for specificity differentiation.[94] He remarks that "it must be remembered that the question is often raised whether chemical properties are indicated by the serum reactions. After the investigations of Obermayer and Pick, Wells and Osborne and ourselves, it might seem superfluous to enter into this matter again, were it not that recently divergent opinions on this point have appeared. Thus the opinion of Traube, who assumes that the chemical nature is without significance for specificity, has found some acceptance, and in a review concerning specificity by Sleeswijk [96] it is considered doubtful if specificity depends fundamentally on qualitative differences. It is clear that such opinions have become untenable. Much more should we take up for investigation the other question, if two substances of quite the same chemical properties can exhibit serological specificity because of physical differences as Pick and Bordet have believed possible."

More recently Landsteiner and van der Scheer [97] have demonstrated that the steric configuration of single groups in an antigen can determine specificity, for they found that antisera against proteins combined with the dextro and levorotatory isomers of an aromatic compound (phenyl paraaminobenzoylamino acetic acid) reacted specifically with the corresponding antigens.

Reaction of Antibodies with Non-antigenic Compounds

Landsteiner [98] also found that if he immunized with a compound of a protein with a non-protein radical, such as metanilic acid (*m*-aminobenzol sulfonic acid), para arsanilic acid (*p*-aminophenylarsenic acid) or *p*-amino benzoic acid, the resulting anti-serum would react with compounds of these radicals with many different proteins, no matter how unrelated the proteins are, e.g., rabbit serum, gliadin from wheat and casein from cow's milk. These results also suggest that specificity depends on single groups in or attached to the protein molecule and not upon the protein molecule as a whole. With such artificial compound proteins, at least, the protein molecule functions only to incite antibody formation, not to determine the specificity, and it owes this antigenic capacity to its large molecular dimensions. *The added groups used in*

[96] *Ergebn. Immunitätsfr.*, 1:395 (1914)
[97] *J. Exp. Med.*, 48:315 (1928)
[98] *Biochem. Z.*, 93:106 (1919).

these experiments are unable of themselves to incite antibody formation, although they can bind the specific antibodies if they are added to the antisera engendered by proteins to which they had been united before serving as antigens.[99] For example, the specific precipitin reaction between a protein treated with metanilic acid and an immune serum made by injecting this compound protein, is inhibited by metanilic acid and closely related sulfonic acids but not by *p*-arsanilic acid, and *p*-amino-benzoic acid, or other unrelated aromatic and aliphatic organic compounds. The converse experiment with proteins treated with *p*-arsanilic acid or *p*-amino-benzoic acid shows the same specificity; e.g., the antiserum for protein treated with *p*-amino-benzoic acid gives precipitin reactions for this protein which are inhibited only by compounds having a carboxyl group attached to the benzene ring. *Apparently these inhibiting radicals can react with the antibodies of the immune serum whether attached to a protein molecule or not, and hence by binding the antibody render it unable to react with the protein antigen containing the radical.* That is, they saturate the specific antibody, although they are unable to produce a precipitation or other observable reaction when not attached to a protein radical. (See also pp. 36-38.) In this respect they differ from the artificial antigens made by mixing lipoids with proteins,[100] for the antibodies to these antigens will react *in vitro* with the lipoids free from the protein part of the antigen. Furthermore, Klopstock[101] has found that diazotized atoxyl mixed with lecithin will give specific complement fixation reactions with an antiserum made by immunizing with diazo-atoxyl blood serum.

Landsteiner made the suggestion that possibly other, non-protein colloidal complexes might, when united with a suitable radical, serve in place of the protein molecule to incite antibody formation, but, as far as we know, such a phenomenon has not yet been demonstrated.

A natural case of specificity depending on the protein radical, while the toxicity depends on the non-protein radical, has been suggested by Nicolle.[102] He calls attention to the fact that according to pharmacological and pathological evidence the poisonous elements of several different sorts of snake venoms must be the same or similar, despite the fact that the protein-rich venom is immunologically specific, the antiserum for one venom failing to protect against another. This may be explained by the assumption that the poisonous element in these venoms

[99] *Biochem. Z.*, **104**:280 (1920).
[100] Landsteiner and Simms, *J. Exp. Med.*, **38**:127 (1923).
[101] *Centr. Bakt.*, Beiheft **104**:140 (1927).
[102] *J. State Med.*, **28**:293 (1920).

is the same, but attached to different and antigenically specific proteins.

Of particular significance is the fact that the experiments of Landsteiner indicate that non-protein substances may not only influence the specificity of immunological reactions but also that under certain conditions these reactions *may give an indication of the nature of a non-protein radical.* For example, it was found that if an antiserum was prepared by immunizing with a compound of para-arsanilic acid with protein, the precipitating power of the serum for this protein complex may be removed not only by saturating it with the homologous compound protein, but also with para-arsanilic acid uncombined with protein, or with other arsenicals containing an aromatic group, but not with arsenicals which do not contain an aromatic group. Similarly, the antiserum for metanilic acid protein may be inactivated by numerous sulfonic acid compounds related to metanilic acid but not by unrelated compounds. Again, an immune serum for para-amino-benzoic acid protein is inhibited only by compounds that also have a carboxyl group in the benzene ring.

According to Sachs, lecithin and cholesterol can be identified by their reactions with specific antisera made against antigens formed by mixing each of these lipoids with proteins, and antipneumococcus sera will serve to distinguish the three different carbohydrate gums of pneumococcus types I, II, and III. This feature of immunological specificity has only recently received attention, and its possible extension cannot now be foretold. The synthetic antigens made by mixing lipoids with foreign proteins differ from the artificial complex proteins described above, which were made by Landsteiner by more energetic union of proteins with simple radicals, in that in the latter case the changes in the protein are so great that homologous proteins become antigenic, whereas with the lipoid-protein antigens the protein must be foreign.[103]

Influence of Physical Properties on Specificity

As Landsteiner has said, the evidence seems clear that chemical changes alone may be sufficient to account for specificity, but that the question is still open as to whether physical properties play any part in determining specificity. Apparently certain physical properties determine whether a substance may serve as an antigen at all—that is, an antigen must be a large colloidal molecule. Can the physical configuration in this molecule determine any of the manifestations of specificity? Among those who have taken the affirmative stand are Pick,[88]

[103] *See* Klopstock and Selter, *Z. Immunität.*, 55:118 and 451 (1928).

who has suggested that physico-chemical properties may come into play in determining the possibility of interaction of antigen and antibody. He believes that the electric charges on the amphoteric colloidal antigen and antibody, and perhaps also their surface configuration and their surface forces, all influence their reaction; these physico-chemical factors greatly complicate the possibility of reaction between two colloids, and to these influences are added the influence of the chemical structure in determining subsequent chemical reactions. It would seem possible that the existence of all these factors may account for specificity, it being necessary for each one of a long series of both physical and chemical adjustments to agree perfectly in order that reaction may take place—just as in a combination lock one lever after another is thrown by the proper manipulation of the dial, and only when all the long series of levers is in just the proper position does the bolt engage and the lock open.[104]

Nevertheless, we still lack a single certain example of specificity determined by purely physical properties (Doerr and Berger).[105] To be sure, denaturation of protein by heat and other means alters their range of specificity,[106] but this process can scarcely be looked upon as a merely physical change. Anson and Mirsky [107] consider denaturation to be a depolymerization, during which some group or groups are uncovered which alter the character of the protein, changing many of its properties, including that of specificity. Freund and Lustig [108] find that in heat coagulation of proteins there are demonstrable chemical changes, including taking up of water, and an increase in free amino groups with resulting increased alkalinity.

Certainly the evidence that the immunological reactions, at least those of agglutination and precipitation, resemble the union of two colloids of different electrical charges (see discussion of Agglutination and Precipitation, Chapter VI) fails to explain specificity. Michaelis and Davidsohn [109] have pointed out that such specific reactions will occur in solutions having a pH of a wide range, indicating that the electrical charge of the components cannot be fundamentally important in the production of specific immunological reactions.

[104] The "resonance theory" of Traube assumes that the surface forces of reacting substances must harmonize, just as the vibration of one tuning fork starts vibrations in another fork only when the two are in harmony, or as electromagnetic waves incite resonance phenomena [*see Z. Immunität.*, 9:246 and 779 (1911)].

[105] *Klin. Woch.*, 1:949 (1922).

[106] Heavy roentgenization does not alter the specificity of proteins [Ling, *J. Cancer Res.*, 9:305 (1925)].

[107] *J. Gen. Physiol.*, 9:169 (1925).

[108] *Biochem. Z.*, 167:355 (1926).

[109] *Biochem. Z.*, 47:59 (1912).

Specificity of Cellular or Tissue Antigens

From the facts mentioned in the previous sections of this chapter, it would seem that in immunization with pure proteins, marked evidences of specificity are obtained which depend on the chemical nature of the proteins. In natural conditions immunological defenses are incited chiefly by more complex cellular antigens, in the form of bacteria and other parasites. Furthermore, artificial immunizations are often carried out with entire cells, especially red corpuscles, and with complex extracts of tissues. With such cellular antigens there are introduced numerous other elements besides proteins, especially lipoids and carbohydrates, which, as pointed out previously, can and do modify the specificity of the resulting antibodies.[110] Hence *in the case of cellular antigens specificity probably depends on the chemical structure of the protoplasm, rather than on the proteins alone.*

Because of the fact that these added agents are usually of simpler chemical character than the proteins, they are more likely to be similar in unrelated species, and so we find oftentimes that interreactions are obtained by cellular antigens from different species; witness the interreaction between one type of Friedländer's bacillus and type II pneumococcus because of similarity in the chemical composition of the gums in their capsule; or the wide distribution of similar "Forssman antigens," presumably because of similar lipoids in unrelated species. Protoplasmic antigens are therefore likely to show in a certain respect less sharp specificity than protein antigens. These considerations help us to apply the excellent comparison of protein and cellular antigens given by Landsteiner and van der Scheer,[111] part of which follows:

"The specificity of proteins in general follows the arrangement of the zoological system, as was shown by the comprehensive experiments of Nuttall on serum proteins, and by the important investigations on hemoglobin by Reichert and Brown. These authors were able to demonstrate that the relationship of the crystal form of hemoglobin parallels to a large extent the zoological relationship of the animals from which the hemoglobin is prepared. The antigens responsible for lysin and agglutinin reactions (i.e., cellular antigens), behave in a different way in that they display sharp differences in related, or even in the same species, and similarities in distant ones. Thus they are not in close correlation with the zoological scale." Examples supporting this view

[110] In the production of hemolysins the antigen seems to be the entire lipoprotein complex, for the stroma proteins will produce only agglutinins and not lysins, according to Locke and Hirsch [*J. Infect. Dis.*, 37:449 (1925)].

[111] *J. Exp. Med.*, 42:123 (1925). *See also* Landsteiner, *J. Immunol.*, 15:589 (1928).

are the substance differentiating individuals of one species, Forssman's antigen, the results of absorption of normal and immune sera with blood of closely related species, and certain results indicating the widespread existence of heterogenetic antigen obtained by absorbing the hemagglutinins of normal sera and separating them from the cells (Landsteiner). Another fact to be mentioned in this connection, is the presence of factors similar to the human isoagglutinogens A and B in animals.

"The assumption that there are present in erythrocytes of different species substances which are similar or identical would seem not to be in keeping with the fact that in most cases immune sera against erythrocytes are specific for the species. Yet it has been shown that an immune serum may not be markedly hemolytic for a certain blood and still contain an antibody active upon a fraction thereof, as in the case described of the flocculation of horse immune sera on rat blood extracts. Furthermore, reactions with high concentrations of immune serum are usually not taken into account, and erythrocytes of only a limited number of species have thus far been examined. Since the whole corpuscles are much more apt to incite antibody production than are extracts, the proteins of the stromata must play an important part in the process of immunization.[112]

"In the attempt to express the foregoing concept one may symbolize the serological structure of erythrocytes (and presumably of other animal cells) somewhat as follows:

"*A, B, C,* etc., represent chemical substances combined with the protein (*P*) or groups contained in such substances. The symbols *Pm* and *Pn* indicate proteins that are nearly identical chemically and serologically. *Pr* is a dissimilar protein of a distant species. According to such a scheme most of the factors (*A, B,* etc.) are the same in closely related species (*m, n*), only a few being dissimilar, while in general the factors of distant species (*m, r*) are different, although identical ones may occur. This view is supported by the fact that when agglutinin

[112] *See* Avery and Heidelberger, *J. Exp. Med.*, 38:73, 81 (1923).

immune sera are absorbed with heterologous blood more of the active substances are taken out by cells of a kindred species than by those of others. Also the individual differences within one species can be accounted for by variations of the factors *A, B;* etc. An idea which may be alternative or supplementary to the first is suggested by the fact that an antibody may react upon several substances not identical, but chemically similar. Accordingly, the occurrence of a certain reaction need not necessarily imply the existence of a special serological factor in every case. Such a view would not require the assumption of a great number of substances in one kind of cells. The possibility that the reacting substances may be present in different amounts has also to be considered. The meaning accorded to the specific factors, *A, B, C,* etc., differs from that embodied in the receptor theory in that the letters signify non-protein chemical substances (or groups of such a substance), separable from the complex antigen, and either not antigenic by themselves or only slightly so (haptens). If the antigen is broken up, as by the action of alcohol, its antigenic power is greatly impaired. According to our conception, species specificity of cells is of a different order as opposed to species specificity of proteins." [113]

Non-specific Reactions

As stated before, there are numerous instances of apparently non-specific immunological reactions, that is, an antiserum for one antigen giving reactions with antigens from sources which seem to be unrelated, at least from a zoological standpoint. Undoubtedly the logical explanation is that there may exist substances in different species which have chemical resemblances or identity, as pointed out above, and this is scarcely to be doubted. We find identical lipoids, fats, nucleic acids, and carbohydrates in different species; many particular types of proteins show apparent chemical identity in different species (e.g., gelatin, keratin); some chemically similar, derived proteins also seem immunologically identical or closely related even when coming from unrelated sources (e.g., lens protein, casein). Therefore, it is probable that tissue proteins may be similar in different species of animal cells, and even in animal and plant cells. Eberson [114] also obtained evidence that different species of bacteria may contain common antigenic proteins as well as specific antigens, accounting for group reactions and non-specific immunization.

[113] For discussion of complex antigens, *see also* Landsteiner, *Klin. Woch.*, 6:103 (1927).
[114] *J. Immunol.*, 5:345 (1920).

Another sort of manifestation of apparently non-specific immunity reactions has been observed especially in therapeutic immunization.[115, 116] Beginning with the classical observations of Matthes that the tuberculin reaction could be produced with deutero-albumose, many similar non-specific reactions have been observed. Particularly the sharp reaction that follows intravenous injections of killed typhoid bacilli into typhoid patients has been found to result equally well if colon bacilli are used, or deutero-albumose. One possible explanation of this type of reaction is that the injected substance acts as a common antigen, which reacts with the common antibodies that were engendered by the antigens of the cause of the disease. Another possibility is that the foreign protein stimulates the tissues that form antibodies so that they produce not only antibodies for this antigen, but also for the antigens of the specific etiologic factor of the disease that have been stimulating the bone marrow previously. Hektoen [117] has observed, for example, that if an animal that has previously produced precipitins for one foreign protein is reinjected with a different protein it will then produce precipitins for both these proteins, and possibly for other proteins with which it has not been injected.[118] It also has been found that animals previously immunized against one type of bacteria are capable of forming more antibodies for some entirely unrelated organism than are control animals.[119] Moreover, the febrile reaction, leucocytosis, increased lymph flow,[120] and other phenomena, such as the altered ferment-anti-ferment balance of the serum (Jobling),[121] which follow injection of non-specific protein, may be responsible for favorably affecting the disease rather than actual antibody formation.

The opposite type of phenomenon, that is, non-specific interference with immunological reaction, or "concurrence of antigens," is illustrated by the observations of J. H. Lewis.[122] He found that small quantities of one protein injected into a guinea pig together with or shortly after large quantities of another protein (e.g., a weak solution of egg albumen in dog serum) would not sensitize the animal, although a similar amount injected alone would always sensitize. The suggested explanation is that the larger amount of foreign protein combines with so many of the

[115] *See* review by Jobling, *J. Am. Med. Assoc.*, **66**:1753 (1916).
[116] Petersen, Wm. F., "Protein Therapy and Non-specific Resistance." Macmillan, 1922.
[117] *J. Infect. Dis.*, **21**:279 (1917).
[118] *See also* Herrmann, *ibid.*, **23**:457 (1918).
[119] Clark, Zellmer and Stone, *J. Infect. Dis.*, **31**:215 (1922); Khanolkar, *J. Path. Bact.*, **27**:181 (1924).
[120] Clark, *Brit. Med. J.*, Feb. 24, 1923, p. 315.
[121] *See* review of this subject by Jobling, Harvey Lectures, **12**:181 (1917); also Cowie and Calhoun, *Arch. Int. Med.*, **23**:69 (1919); also Petersen,[116]
[122] *J. Infect. Dis.*, **17**:241 (1915).

available cell receptors that few of the small number of sensitizing protein molecules are able to be bound to the cells and to stimulate antibody formation; this explanation assumes a certain lack of specificity on the part of the cell receptors.

An interesting illustration of the fact that whatever stimulates the bone marrow may cause it to form, among other blood elements, specific antibodies, is furnished by the behavior of antitoxin-producing horses. If a horse that has been immunized to diphtheria toxin is bled as much as possible, it will be found to have regenerated the lost antitoxin within 48 hours,[123] although the last immunizing dose of toxin was received long before, presumably because bleeding powerfully stimulates the bone marrow to regenerate the lost blood elements. Also, it is stated that persons who have once had typhoid, but whose blood no longer contains much agglutinin, may show a high typhoid agglutinin content when infected by some other organism, or after any sharp febrile attack. It is highly possible that many therapeutic agents may similarly act by stimulating the tissues to increased formation of specific antibodies, e.g., arsenic, mercury and other metals,[124] heliotherapy, hemorrhage [125] or phlebotomy, hot baths.

Recapitulation

Immunological specificity is but one of the innumerable manifestations characteristic of all biological processes, from the fertilization of the ovum by a specific spermatozoon on through the development of specific structures and the specific cellular manifestations characteristic of each species. The study of specificity by immunological methods has thrown light on the entire subject of the biological specificity, and demonstrates it to depend chiefly on the chemical structure of the proteins, often at times modified by carbohydrate and lipoid complexes. But only the proteins are antigenic, and, as far as now known none of the other constituents of the blood and tissues can account for the infinite variety of the manifestations of specificity, for they are limited in number and most of them are quite the same in many or all different species. The variety possible in the proteins is practically unlimited because of the number of different combinations that can be made with the score of amino acids found in them. It has been found that the immunological differences that exist between different species of bac-

[123] O'Brien, *J. Path. Bact.*, 18:89 (1913).
[124] *See* Walbum, *Ann. Inst. Pasteur*, 37:396 (1923); *Z. Immunität.*, 43:433 (1925); Schmidt, *Z. Immunität.*, 45:305 (1925).
[125] *See* Hahn and Langer, *Z. Immunität.*, 26:199 (1917); Trommsdorf, *ibid.*, 32:379 (1921).

teria, between extracts of different plants, or between the blood and tissues of different species of animals, are dependent on differences in the composition of their proteins whenever isolated proteins are under observation. In the case of certain bacteria the differences between species seem to be determined by the proteins, whereas the difference between types within a species is determined by complex but non-antigenic carbohydrates.

As far as investigations have been made, any two proteins that are identical immunologically are indistinguishable chemically, those that are readily distinguished by one of these methods are also readily differentiated by the other. Proteins that are shown to be related but still distinguishable by immunological tests are found to be similar to one another in chemical composition, but nevertheless show recognizable chemical differences when suitable methods are used. The same is true of bacterial carbohydrates as far as yet investigated.

Biological specificity depends chiefly on chemical individuality of proteins, and biological relationship is equally associated with the presence of chemically similar proteins. The lipoids and carbohydrates of protoplasm may also play a subordinate rôle in determining immunological specificity. When whole cells, or extracts containing most of the constituents of cells are used, the antigen is *protoplasm,* and in this case the specificity is determined not only by the proteins but by the modifying elements of the protoplasm, especially the lipoids, sometimes the carbohydrates, and possibly other components.

A single species of animals, or even a single animal, contains many different proteins which may be distinguished immunologically and chemically. A single protein, likewise, may be found widely distributed through many different species, its identity being established readily by immunological methods, and equally certainly but less easily by chemical procedures. Although the antigenic capacity of a protein depends on the entire large colloidal molecular structure, its specificity seems to reside in certain of the radicals of the molecule. There is evidence that a single protein may exhibit more than one specific immunological reaction, by virtue of possessing more than one such active radical. Group reactions exhibited by complex cellular antigens from biological related species may therefore depend either on the presence in these antigens of both common and specific proteins, carbohydrates, and lipoids, or by the presence in different proteins of common and specific reactive radicals.

The immunological specificity of proteins may be artificially modified

by introducing into them various radicals. It is then found that a single group in the added radical may by itself affect the specific immunological behavior of the entire molecule. Also, not only the character of this specific group is of influence but also its place in the added radical (Landsteiner), indicating that spatial relations are of importance in determining specificity. Apparently an antibody can react with various related but not identical antigens, specificity being always a quantitative matter which reaches its maximum when the antigen is reacting with an antibody produced by immunizing with an identical antigen. It is not yet definitely determined whether purely physical alterations in antigenic proteins are associated with alterations in specificity, for the protein molecule is so labile that any physical alterations are likely to be associated with chemical changes. As yet, however, we have no proved case of immunological specificity determined by purely physical properties.

Non-specific immunological reactions may depend on the existence of identical proteins, carbohydrates, and lipoids, in different species, or of different proteins with identical groupings or bound to identical lipoids or carbohydrates, determining a common specificity. Also, the stimulation of the mechanism of antibody formation by one antigen (A) may arouse the production of antibodies for other antigens (B and C), especially if the individual has previously developed a capacity to produce antibodies for these antigens (B and C).

CHAPTER IV

THE NATURE OF THE ANTIBODIES

After immunization with a given antigen, whether artificially or through the natural processes of infection, the blood of the animal, and possibly all the fixed tissues as well, usually come to exhibit the capacity to react in some way or other with the antigen, in a manner qualitatively different or in a degree quantitatively greater than previously. We attribute this altered reactivity to the presence of "antibodies," despite the fact that we have absolutely no knowledge what these antibodies may be, or even that they exist as material objects. Like the enzymes, we recognize them by what they do without knowing just what they are. We do not know whether they are specific molecular aggregates or merely physical forces dependent on altered surface energy of the same substances which were already present in the blood before the process of immunization was ever begun. Their existence as, or associated with, tangible specific material is shown by the fact that something with the antibody property can be removed from the immune serum by adsorption to the specific antigen, and that this adsorbed agent can be readily split from the antigen in a concentrated, active form.

The methods for their recognition are several, and according to the procedure employed we designate the antibodies as precipitins, agglutinins, antitoxins, complement fixation antibodies, opsonins, cytolysins, anaphylactins, and so on. These names assume the existence of several different and distinct reactive substances or antibodies, and this assumption has been currently accepted as if it were an established fact, although this is far from the case. Of late there has been a growing tendency to doubt that there is any such considerable variety of antibodies, for we usually find after immunizing even with a single purified protein as antigen that we can demonstrate several if not all of these properties in the serum of the immunized animal.

Are There Different Types of Antibodies?

An attractive hypothesis is that there are two fundamental types of immunity reactions.[1] One, having to do with substances which are

[1] Zinsser, *Arch. Int. Med.*, 16:223 (1915).

essentially active poisons, neutralizes or inhibits their toxicity by direct chemical action. In this group come the antitoxins for bacterial toxins (diphtheria, tetanus, etc.), and the antibodies for venoms, vegetable toxins (ricin, abrin, etc.) and various bacterial hematotoxins. It is to be noted that these toxic substances, the true toxins, are all similar to one another in being classed as large colloidal aggregates, resembling proteins, but not yet finally identified as proteins.

The other group of immunity reactions is concerned with defense against foreign proteins or foreign protoplasm, whether toxic or non-toxic and whether in solution or aggregated into cells (bacteria, corpuscles, tissue cells). In all the reactions of this group we deal with processes that tend to alter the colloidal state of the foreign proteins, by making them larger aggregates (precipitation, agglutination), or smaller aggregates (proteolysis, hemolysis, bacteriolysis, cytolysis), and in each case the reaction consists of two separable steps, sensitization and reaction. It is tempting to accept the view, championed especially by Friedberger, Zinsser, Dean, and Nicolle, that in this second group of reactions but one and the same antibody is concerned, and that all the reactions are essentially the same, differing merely in the method by which the reaction is demonstrated. There are, indeed, many facts capable of interpretation as supporting this hypothesis, but there are still other observations that do not harmonize with it, notably the lack of constant quantitative relations between the different reactions produced by the same immune serum, and as yet it is neither completely established nor disproved.

Evidence Favoring Unity of Antibodies

Zinsser has christened this the "unitarian" hypothesis, and presented much of the evidence in its favor. It is indeed a fact that the identity of precipitin and agglutinin has been commonly accepted, it being recognized that the precipitin reaction is obtained when the colloidal particles of a dissolved antigen are brought together into aggregates too large to remain suspended, and that the agglutinin reaction occurs when the colloidal antigen is already in large undissolved masses which also are brought together and precipitated. As Zinsser [2] says:

"If the antibody comes in contact with a very finely divided antigen, as in a bacterial extract or in, let us say, horse serum, if electrolytes are present and perhaps other necessary physical factors furnished by the presence of serum, etc., precipitation occurs.

[2] "Infection and Resistance," 1923, p. 319.

"When we are dealing with whole bacteria of relatively large mass and correspondingly small surface exposure, agglutination is the result, and quantitative parallelism with the precipitin reaction is not to be expected because of the much greater dispersion of the antigen in the latter test.

"When alexin is present complement fixation or hemolysis or bactericidal effects result, since the changes produced by the sensitization have permitted union with the complement.

"When there are leucocytes present the union makes possible the phagocytosis of the antigen, and when the antibody is absorbed by the cells of an animal, anaphylactic 'sensitization' occurs."

The chief fact which led to the view that there are as many different antibodies as there are ways of demonstrating them, was the common observation, especially in the early days before the best technical methods were developed, that an antiserum might exhibit marked quantitative differences in its activity in respect to the different sorts of reactions when tested with the same antigen. Sometimes, indeed, one reaction could be demonstrated when another could not be obtained at all. In immunizing with soluble protein antigens it is usually observed that the complement fixation reaction may first be demonstrated, but that later, when good precipitin reactions are obtained, the serum also exhibits the capacity to sensitize animals to the antigen, or, in the nomenclature of immunology, the precipitins and the anaphylactins appear together. This and other observations have given much support to the doctrine that the precipitin and the anaphylactic sensitizing antibody are one and the same. The relative proportion of these two antibodies is usually found to run parallel in immune sera, and Richard Weil [3] added much in support of their identity by finding that the precipitate obtained when immune serum reacted with the specific protein, if injected into guinea pigs, conferred passive sensitization to the specific antigen. Since the precipitate formed in the precipitin reaction is known to consist chiefly if not entirely of precipitin, this experiment indicates that probably the precipitin is also the immune body responsible for the anaphylaxis reaction.

Felton and Bailey [4] also found that an isolated and repurified protein antibody obtained from antipneumococcus serum exhibited agglutinin, precipitin, opsonin, complement fixation, and protection reactions, thus supporting the hypothesis that a single antibody accomplishes all the

[3] *J. Immunol.*, 1:19 (1916).
[4] *J. Immunol.*, 11:197 (1926).

known immunological reactions. That there should not always be a quantitative parallelism between the complement fixation antibodies and the precipitins is no argument against their identity, and there is much evidence in support of the assumption that both these reactions depend on the same antibody.[5] As Dean has pointed out, the reason why the two reactions do not run a parallel course is not that they are caused by two different sets of antibodies, i.e., precipitins and amboceptors, but because they represent two phases or two stages of the same reaction, and it may not be possible to demonstrate both stages under the same conditions.

Went [6] found also a parallelism between agglutinin and opsonic activity of immune sera, which he interprets as indicating that both depend on the same antibody.

There is also a growing tendency to suspect that even antitoxins and precipitins are identical, since Ramon [7] developed a method for assaying the strength of diphtheria antitoxic sera by determining its capacity to produce precipitin reactions with diphtheria toxin. However, it is not yet fully established that the precipitate formed when toxin and antitoxin are brought together in proper proportions represents a specific precipitin reaction between toxin and the antitoxin,[8] rather than between other elements in these complex mixtures (see p. 134 for further discussion). It seems to be well established at least that the precipitins and agglutinins for toxin-producing bacteria are distinct from the antitoxins that neutralize their toxins.

Significance of Quantitative Discrepancies

The failure to secure parallelism in titration of the several antibodies in a given serum lacks force as negative evidence against their identity in the face of the known great quantitative inaccuracy of most immunological measurements, and the ready inhibition of the reactions by factors which are often unknown, for the variables in all these tests are far too many and too uncontrollable to permit of exactitude. Thus, as Zinsser points out, we must not forget that agglutination and precipitation are actually only secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon a great many environmental factors which may not, to the same degree, influence

[5] This relation is considered more in detail in the discussion of complement fixation, Chapter VII.

[6] *Z. Immunität.*, 39:76 (1924).

[7] *Ann. Inst. Pasteur*, 38:1 (1924).

[8] Such precipitating mixtures will also fix complement [*see* Dean, *J. Path. Bact.*, 30:675 (1927)].

phenomena in which alexin (complement), the leucocytes or the body cells of animals are involved. The flocculation reactions depend upon the presence and the concentration of electrolytes, upon the pH, upon mutual relations of concentration, and perhaps upon viscosity. The suspension equilibrium of the sensitized antigen must to some extent depend upon the varying factor of the inactive serum constituents carried into the union with the antibody, for we know that in precipitation reactions the bulk of the precipitate comes from the immune serum, and yet antibodies relatively free from protein can be split off from such a complex; this proves that, in the union, much inactive protein substance is carried along, which inevitably must influence reactions of flocculation.

It is not to be wondered at, Zinsser says, that in view of the factors mentioned above, agglutination and precipitation curves should not run parallel with the curves of other antibody functions. In regard to such lack of parallelism, while it has frequently been seen that agglutinating and precipitating functions are often weaker than other antibody effects, or even absent entirely in such sera, it has rarely been observed that they are powerfully and specifically present when other effects are lacking.

He concludes, "We do not wish by any means to convey the impression that we consider the 'unitarian' view as absolutely and rigidly proved. We do believe, however, that the denial of such a view necessitates the assumption that the injection of a pure antigen calls forth five or six fundamentally different reactions on the part of the tissue cells, a theory which would be justified only on the basis of incontrovertible proof."

Unity of Antibodies Agrees with Bordet's Theories

The ideas of the French school of immunology, sponsored by Bordet, naturally fit into the unitarian hypothesis of the antibodies, for instead of recognizing the existence of definite antibodies as postulated by Ehrlich, they have always sought to explain the phenomena of immunity as the result of simple physico-chemical reactions between the colloids of the serum and the antigen. These views are reviewed by Nicolle,[9] who holds that there is but one sort of antibody, and this has the property, as first shown by Bordet, of rendering the antigen susceptible to coagulation by the electrolytes present in the mixture, constituting the agglutinin and precipitin reactions, as well as one phase of

[9] *J. State Med.*, 28:293 (1920); "Antigens et les Anticorps," Masson et Cie, Paris, 1920; also Nicolle and Césari, *Ann. Inst. Pasteur*, 36:463 (1922).

the opsonic and anaphylactic sensitizations. If complement is also present in the mixture we get the lytic phenomena of immunity, i.e., hemolysis, bacteriolysis, cytolysis, and possibly the formation of the anaphylactic poison. When the coagulation reaction is especially vigorous the aggregation of the antigen into dense masses seriously interferes with the lytic phenomena, through reducing the surface for attack by the enzymes; this explains why a strong precipitin serum may seem to be weak in lytic activity, without postulating the existence of separate antibodies. Nicolle would even include the toxin-antitoxin neutralization in the same class. He maintains that true toxins and their specific antitoxins precipitate each other, and has based on this principle methods of titration of antigens and antibodies.[10]

Objections to the "Unitarian" Hypothesis

Despite all the arguments that may be advanced in favor of the "unitarian" hypothesis, at least as covering the antibodies for protein antigens other than true toxins, it is by no means universally accepted, for arguments of more or less weight have been advanced against it. Dean [11] has called attention to the fact that in active tuberculosis the serum shows a decrease in opsonins which sensitize tubercle bacilli to phagocytosis, while at the same time there is an increase in the complement fixation power of the serum. This would suggest that these antibodies, opsonins and complement-binding amboceptors, are not the same, but it is possible that there is some other explanation for the discrepancy; for example, in active tuberculosis substances may appear which interfere with phagocytosis or which prevent the effect of opsonins on tubercle bacilli without interfering with complement fixation.

Hektoen [12] has observed opsonins in the spinal fluid of dogs when agglutinins could not be demonstrated, although the blood of the animal exhibited both opsonic and agglutinative activity. This finding suggests that the opsonin had been selectively secreted or filtered into the spinal fluid, while the agglutinin had been held back, supporting the view that these are distinct antibodies.

Antibodies are inactivated by heat in a rather wide range, between 65° and 85°, varying with different sera, but there is no constant difference to be observed between the different types of reactions in this respect.[13] However, Forssman [14] has reported that when anticorpuscle

[10] *Compt. rend. Acad. Sci.*, 169:1433 (1919).
[11] *Brit. Med. Jour.*, Dec. 1st (1923).
[12] *J. Infect. Dis.*, 12:1 (1913).
[13] *See* Jones, *J. Exper. Med.*, 46:291 (1927).
[14] *Compt. rend. Soc. Biol.*, 86:495 (1922).

serum mixed with ether is heated at 56° the hemolysin is destroyed while the agglutinin is unaffected.

Landsteiner [15] has pointed out that there are peculiarities in the specificity manifested by agglutinogens and precipitinogens which suggest that there is an essential difference in the chemical structures which determine the specificity of the two kinds of antigens, and hence presumably the antibodies must also differ. There are also such observations as that of Mackie [16] who found that a highly potent agglutinating serum obtained by immunizing with a certain strain of colon bacilli did not agglutinate other strains of colon bacilli which appeared identical with the antigenic strain by every other test; neither did these heterologous strains of colon bacilli absorb the agglutinins. But this same agglutinating antiserum gave complement fixation reactions with many strains of colon bacilli, even when these were in other respects very different from the original antigenic strain. These results suggest the existence of some distinct difference between agglutinins and complement fixation antibodies. Furthermore, Singer [17] reports the separation of hemolytic amboceptors from the hemagglutinins in an immune serum, and Coulter [18] states that partial denaturation with alcohol of the antibody-containing protein of an antierythrocyte serum, destroys the hemolytic activity while the agglutinating activity is increased. Also there is the observation of Locke and Hirsch [19] that while lipin-free stroma protein may be antigenic in the production of hemagglutinin, it requires the entire lipo-protein complex to produce hemolysin.

One fact that may be advanced as failing to harmonize with the unitarian hypothesis, is the observation, repeatedly made, that the different antibodies are contained in different fractions of the serum proteins. It has been found by some observers that antitoxin is in the water-soluble globulin fraction or pseudoglobulin, along with opsonins, hemolysins and antibacterial immune bodies, while in the euglobulin (water-insoluble) fraction have been found the precipitins, agglutinins, anaphylactins, and complement fixation antibodies. However, these findings are far from convincing for two reasons:

(1) The results of different investigators do not agree as to which fraction contains which antibody. For example, Doerr and Hallauer found that in separation of the proteins of immune serum by electrodialysis, the precipitin and anaphylactin were found to be sometimes in

[15] Landsteiner and van der Scheer, *J. Exp. Med.*, **40**:91 (1924).
[16] *J. Path. Bact.*, **18**:137 (1913).
[17] *Z. Immunität.*, **35**:191 (1922).
[18] *J. Gen. Physiol.*, **10**:545 (1927).
[19] *J. Infect. Dis.*, **37**:449 (1925).

one fraction alone, sometimes equally divided in both globulin fractions, but always inseparable; whereas Otto and Iwanoff [20] sometimes found the precipitin solely in the pseudoglobulin while the anaphylactin was divided between the two globulins. Again, Ruppel [21] found the hemolytic amboceptor in the pseudoglobulin and the complement fixation antibodies in the euglobulin, although these are ordinarily considered as identical. He considers all the antibacterial protective elements of serum to be in the pseudoglobulin fraction, but Homer [22] found the antidysenteric and antimeningococcic agents of the specific immune serums to reside chiefly in the euglobulins. By electrodialysis, Locke and Hirsch [23] found hemolytic amboceptor in the pseudoglobulin; whereas, by precipitation methods, Felton [24] found the protective antibody of antipneumococcic serum in the water-insoluble globulins.

(2) The chemical nature of pseudoglobulin and euglobulin is not well defined. Chemical and physical studies have shown no appreciable difference between the two unless it be the presence of lipoids in the euglobulin fraction, although immunological tests show them to be different from one another. Ruppel [21] and others claim that pseudoglobulin tends to pass over to the euglobulin fraction, or at least to become less and less water-soluble, so the purity and constancy of these fractions is open to question.[25] But in any case the antibodies seem definitely associated with the globulins rather than the other proteins of the serum, and Dean maintains that in all the serum reactions there is an aggregation of globulin particles about the antigen.

Antibodies as Altered Antigens

Not a few investigators have suggested that antibodies are merely altered antigens. For example, Herzfeld and Klinger advanced the hypothesis that fragments split from the antigens unite with other colloidal particles to constitute the antibodies, which owe their specific property to the fact that they contain fragments of the antigen with which they react.[26] Locke, Main and Hirsch [27] also found indication that in hemolytic sera there is a substance, probably derived from the

[20] *Z. Immunität.*, **54**:496 (1928).
[21] *Deut. med. Woch.*, **49**:40 (1923).
[22] *Biochem. J.*, **14**:42 (1920).
[23] *J. Infect. Dis.*, **35**:519 (1924).
[24] *J. Infect. Dis.*, **37**:199 (1925).
[25] *See* Mona Adolf, *Klin. Woch.*, **3**:1214 (1924).
[26] A somewhat similar hypothesis has been proposed by Manwaring [*Scientific Monthly*, **25**:362 (1927)]. Smirnow [*Beitr. klin. Tuberk.*, **59**:278 (1924)] reports that he has produced antitoxin by treating old bouillon cultures of diphtheria by electrolysis with oxidation, and believes that in the body a similar effect is produced by enzymes.
[27] *J. Infect. Dis.*, **39**:126 (1926).

antigen, upon which is adsorbed some naturally-occurring protein substance, this large complex constituting the specific amboceptor.

Against such hypotheses may be advanced the fact that if an immunized animal is bled, the titer of antibodies present in the blood left in the animal falls temporarily, and then rises, often to a point even higher than before, despite the lack of antigen to furnish the hypothetical nuclei for the new formed antibodies.[28] The attempts reported to produce antibodies *in vitro* by the action of antigen on serum (e.g., Mez and Ziegenspeck), have not been generally accepted as establishing such a possibility.[29]

The Nature and Properties of Antitoxins

Accepting the prevailing view that the antitoxins are different from all the other antibodies, in that they manifest the property of specifically neutralizing certain types of poison in a quantitative manner, we may consider what is known about them. Because of their great practical importance they have received much more study than the other antibodies, and enough has been learned of their properties to permit of considerable concentration of the antitoxic activity of sera. It is generally agreed that the antitoxins are associated chiefly if not entirely with the water-soluble pseudoglobulin fraction of the serum. Adolf [25] has found that if antitoxic serum is subjected to electrodialysis until the conductivity is reduced to that of distilled water, the antitoxin disappears entirely, only albumin being left in solution and this is free from antitoxin. This experiment raises doubt as to the existence of a true water-soluble "pseudoglobulin" carrying the antitoxin, but it was found that during such dialysis the antitoxin comes out with the latter part of the precipitated globulin, which undoubtedly corresponds to the globulin fraction commonly considered as pseudoglobulin whether it is truly water-soluble or not. But we do not know whether the antitoxin is a definite substance adherent to the pseudoglobulins, or a form of pseudoglobulin itself which differs from normal pseudoglobulin in some change in physical properties or addition of active radicals, whereby it has obtained the capacity of neutralizing toxin.

In any event the antitoxins behave as amphoteric colloids, migrating to the cathode or anode according to the pH of the solution (Maver and Falk),[30] diffusing little or not at all, their reaction curve resembling

[28] From editor's note in French translation of 1st Edition of this book, p. 132.
[29] *See* Eisler, *Z. Immunität.*, 53:136 (1927).
[30] *J. Immunol.*, 14:219 (1927); also Maver, "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928.

more an adsorption curve than the reaction curves of crystalloids, and being influenced by all conditions that influence colloids.[31] Whether the active groups (receptors) are secreted in a free condition in antitoxin formation, or combined with a large molecule, is unknown.

It is an interesting fact that the antitoxins formed by different animals which have been immunized with a given toxin seem to be the same—horse serum, or sheep serum, or goat serum will neutralize diphtheria toxin if the animals have been made immune to this toxin;[32] and, furthermore, their serum when introduced into the body of an entirely different animal, e.g., a guinea pig or a child, will neutralize diphtheria toxin within its body. Equally important is the fact that the antitoxin for one toxin will not neutralize any other toxin; e.g., diphtheria antitoxin will not neutralize tetanus toxin, or conversely, but we have not the slightest idea just what chemical or physical difference determines this specificity of the antitoxins.

According to Eisler,[33] toxin injected in a single moderate sized dose produces about the same amount of antitoxin as the same amount divided into several injections, although the less soluble antigens notoriously give much more antibody formation if they are injected in several small doses (Maisin),[34] provided the subdivision is not carried too far.[35]

Are Antitoxins Globulins?

The fact that during immunization of an animal the proportion of pseudoglobulin in the blood is increased [36] does not establish that antitoxin actually is this new-formed globulin, for Meyer [37] and his colleagues found in their study of the blood proteins during immunization, that the proportion of globulins increases according to the severity of the intoxication, and not in any definite relation to the degree of immunity or antitoxin production.[38] Not only bacterial poisons but also any protein antigen causes an increase in the globulin content of the immune serum (Doerr and Berger, lit.),[39] but if the immunization is carried out carefully with small quantities of antigen there may be

[31] For full bibliography on the chemical properties of antitoxic sera and antitoxins *see* review by Crawford and Foster, *Am. J. Pharm.*, **90**:765 (1918).
[32] Perhaps when we get away from the mammals the antibodies engendered by the same antigen may differ [*see* Levaditi and St. Mutermilch, *Ann. Inst. Past.*, **27**:924 (1914)].
[33] *Centr. Bakt.*, Abt. 1, **79**:291 (1917).
[34] *Reunion soc. belg., biol.*, p. 1575, (1920).
[35] *See* Friedberger and Seidenberg, *Z. Immunität.*, **52**:202 (1927).
[36] *See* Kirkbride and Murdick, *J. Immunol.*, **14**:235 (1927).
[37] *J. Infect. Dis.*, **22**:1 (1918).
[38] For review on the quantitative relations of serum albumin and globulin, *see* Lovett, *Arch. Pathol.*, **4**:984 (1927).
[39] *Z. Hyg. Infekskr.*, **93**:147 (1921); Perger, *Z. ges. exp. Med.*, **28**:1 (1922).

no increase of the globulin (Glaesner),[40] indicating that antibody increase is not essentially associated with globulin increase, nor is the relation of globulin and antitoxin increase a constant one.[41] The average antitoxic horse serum contains 12 per cent albumin, 78 per cent of soluble globulin containing the antitoxin, 10 per cent euglobulin; whereas in normal non-immunized horses the proportion is 40 per cent albumin, 42 per cent pseudoglobulin and 18 per cent euglobulin. Homer [22] found that there seemed to be a slight difference between diphtheria and tetanus antitoxin, more of the latter being in the portion of the protein in the pseudoglobulin-euglobulin zone. As far as we know the only attempt made to determine whether the antitoxin-containing pseudoglobulin is chemically different from normal pseudoglobulin showed no very distinct differences, although the histidine nitrogen figure seemed to be lower.[42]

Although the immunization of an animal is usually accompanied by a marked rise in the proportion of globulin in the serum, studies of the physical properties of the serum have not shown much change that could be correlated with the presence of specific antibodies. Du Noüy [43] found no change in the refractive index of the serum in immunized animals, but did observe a difference in the effect of time on the surface tension. When serum is diluted the drop in surface tension which normally takes place on standing becomes more marked, and this "time drop" shows a maximum in certain dilutions, usually about 1 to 10,000. Immune serum shows 50 to 100 per cent greater time drop than before immunization. It is not dependent on a change in the albumin-globulin ratio of the serum.[44] Immunization also causes a special form of crystallization of added sodium chloride when the serum evaporates.[45] The meaning of these changes is not known, beyond indicating that there is "in certain immunity states a decrease in the surface energy of the substances normally absorbed in function time in the surface layer." Neither is it known whether these physical changes are related to the formation of antibodies, and Du Noüy found that the mean length of the serum protein molecules is not appreciably different after immunization.[46]

A difference in the absorption band between the wave lengths 2950

[40] *Z. exp. Path. Ther.*, **2**:154 (1906).
[41] Reymann, *Z. Immunität.*, **39**:15 (1924).
[42] Banzhaf, Sugiura, and Falk, *J. Immunol.*, **2**:125 (1916).
[43] *J. Exp. Med.*, **37**:659 (1923). Also, "Surface Equilibria of Colloids," Du Noüy, Chemical Catalog Company, New York, **1926**.
[44] Du Noüy and Baker, *J. Exp. Med.*, **42**:9 (1925).
[45] Du Noüy, *J. Exp. Med.*, **41**:779 (1925).
[46] *Biochem. Z.*, **165**:134 (1925).

and 2400 has been observed in the serum of different species of animals when immunized,[47] but the significance of this change is also unknown.

An interesting hypothesis advanced by Ostromuislenskii[48] is that antitoxin is merely normal serum globulin physically altered by the toxin which it has adsorbed. He claims that normal serum globulins combine with toxins, and if after they have been thus united for some time they are dissociated by the action of acids, free specific antitoxin may be obtained. If this is true it should be possible to prepare antitoxins *in vitro* without animal immunization, and the practical importance of this suggestion entitles it to further consideration.

The Resemblance of Antitoxins to Proteins

The relation of antitoxins to proteins has also been investigated by permitting digestive enzymes to act on antitoxic serum. Pick digested the antitoxin-containing globulin of horse serum for several days with trypsin; after five days, when part of the protein was still not digested, the antitoxin was but little impaired in strength; after nine days, when most of the protein was digested, the antitoxin had lost two-thirds of its strength. This indicates a considerable resistance of antitoxin to trypsin, but also shows that it is affected in much the same way as the globulin (which is itself very resistant to trypsin) and therefore is presumably of similar nature. Antitoxin seemed to be much more rapidly destroyed by pepsin-HCl digestion than by trypsin, in which respect it again resembles the serum globulin. Berg and Kelser[49] found that trypsin and pepsin destroy the antitoxin and serum proteins at about the same rate, but their failure to observe "significant chemical changes" in the proteins of serum acted upon by weak acid or alkali that slowly inactivate antitoxin, does not seem to warrant their deduction that antitoxin is non-protein.

In favor of the view that antitoxin is a definite protein body or attached to one, is the fact that it is not carried down in indifferent precipitates, as are the enzymes, but comes down always in a certain fraction of the protein precipitates, e.g., we can precipitate all the serum albumin from an antitoxic serum, and it does not carry down with it any of the antitoxin. Another important point has been brought out by Arrhenius and Madsen,[50] who determined approximately the molecular weight of toxin and antitoxin by means of their rate of

[47] Tadokoro and Nakayama, *J. Infect. Dis.*, **26**:8 (1920).
[48] *J. Russ. Phys. Chem. Soc.*, **47**:263 (1915).
[49] *J. Agric. Res.*, **13**:471 (1918).
[50] Festskrift Staatens Serum Institut, 1902.

diffusion, and found that the toxins (diphtheria toxin and tetanolysin) diffused ten or more times as rapidly as the corresponding antitoxins. Gelatin filters also hold back antitoxin and let toxin pass through, and toxins diffuse into cells which seem to be impermeable for the antitoxin. This indicates that the antitoxin molecules are much larger than the toxin molecules, agreeing with the idea that antitoxin is of protein nature and that toxin either is not protein or is smaller than most protein molecules. It is difficult, however, to accept the figures of Arrhenius,[51] which give the size of the antitoxin molecule as 100 times as great as that of the toxin molecule, which in turn may have a molecular weight of 15,000.

Ramon found that diphtheria antitoxin could be concentrated by collecting the precipitate it forms with toxin, and dissociating the toxin and antitoxin with weak acids at 60°. By isoelectric fractionation Locke and Main [52] secured the antitoxin in exceptionally pure form, and found that it exhibits the immunological properties of horse serum protein and does not withstand boiling.[53] Another point in favor of the protein nature of the antitoxins is that precipitin reactions with antitoxin serum throw down the antitoxin in the precipitate, and may even make the antibody inactive when there is no visible precipitate (Eisler).[54] The same is true of other antibodies.[55]

Taken all together, *the evidence indicates even closer resemblance or attachment of antitoxins to proteins than has been shown for the toxins, and all attempts to separate antitoxins from proteins have so far failed.*

Physical Properties of Antitoxins

Antitoxins are retained to greater or less extent by porcelain filters, do not pass through dialyzing membranes readily, and are in general easily destroyed by chemical and physical agencies, although much less so than are most toxins. Heating to 60°-70° injures, and boiling quickly destroys them, although like the enzymes and the proteins, they resist dry heat to 140°, and also extremely low temperature, without change. If antitoxic serum is heated 12 hours at 57° a considerable part of the soluble globulin becomes insoluble without a corresponding loss of

[51] *Z. Chemother, Ref.*, 3:423 (1914).
[52] *J. Infect. Dis.*, 39:484 (1926).
[53] Salkowski [*Biochem. Z.*, 132:84 (1922)] reported in 1922 that in 1896 he had obtained a small proportion of the antitoxin of a serum, about 20 per cent of the original titer, in a protein-free form by precipitating the proteins with NaCl and trichlor-acetic acid. A repetition of this work by Dold and Freudenberg [*Biochem. Z.*, 162:169 (1925)] failed to confirm it.
[54] *Centr. Bakt.*, Abt. I, 84:46 (1920).
[55] Landsteiner and Prasek, *Z. Immunität.*, 10:68 (1911).

antitoxin (Banzhaf). Apparently the antitoxic globulins have somewhat smaller or more stable molecules than most of the rest of the serum globulins, which permits of their partial purification for therapeutic purposes.

Diphtheria antitoxin is destroyed by heat of varying degree (65°-74°) at rates which correspond to the theory for a bimolecular process (Madsen).[56] Apparently heat inactivation depends on coagulation of the serum proteins, since when agents are added to immune serum to prevent coagulation, no inactivation occurs on heating to temperatures far above the usual thermal destruction point.[57] However, the changes in viscosity or coagulation of serum proteins under the influence of heat do not follow the same laws as the antitoxin destruction. At lower temperatures (5°-25°) the temperature coefficient of the destruction of antitoxin is but one-tenth as great as at the higher temperatures. The chief significance of these and related observations on the physicochemical behavior of antibodies would seem to be that they indicate that in their properties and their reactions the antibodies follow the same laws as are applicable to known chemical phenomena.

Antitoxins can be preserved for a very long time when dried completely, but in the fluid serum they gradually disappear, especially if exposed to light and air. The more the antitoxin is purified by removal of inert serum proteins the more stable it is.[58] Acids and alkalies destroy antitoxins, acids being the more harmful in low concentrations. Like the enzymes, antitoxins are destroyed by ultra-violet rays. They are destroyed in the alimentary tract, without appreciable absorption except in the case of new-born animals suckling mothers whose blood and milk contain antitoxin.[59] After subcutaneous injection, antitoxin soon disappears from the blood; part may be bound to the tissues, part may be destroyed, since only traces appear in the urine.

Eisler[60] studied the adsorption of antitoxin by animal charcoal, which renders the antitoxin entirely unable to unite with toxin, at the same time reducing the capacity of the charcoal to bind toxin. Although the antitoxic power of an immune horse serum is thus removed by charcoal, yet the protein so fixed to the charcoal is still capable of giving precipitin and complement fixation reactions with antibodies for horse serum. Hence the charcoal has apparently fixed more firmly the antitoxic elements of the horse serum than at least some of the proteins of the

[56] *J. State Med.*, **31**:151 (1923).
[57] Silber and Shaechter, *Z. Immunität.*, **53**:263 (1927).
[58] Personal communication of observations by P. G. Heinemann.
[59] Famulener, *J. Infect. Dis.*, **10**:332 (1912).
[60] *Biochem. Z.*, **135**:416 (1923).

serum, which are still sufficiently free to react with antibodies. It has also been shown that after antitoxin has been bound to toxin within the serum it still reacts with precipitins, and if the precipitin reaction is performed soon enough after the neutralization the toxin is set free from its union with the antitoxin. The adsorption of antibodies by charcoal would seem to be a very delicate and particular sort of process, for different samples of antisera containing the same specific antibody may have it adsorbed in quite different quantities. Probably the concentration of protein and other substances in the serum, especially the H-ion concentration, affects greatly the adsorption of the antibodies.

The Nature of the Amboceptors and Other Antibodies

Our ignorance of the exact chemical nature of the most studied class of antibodies, the antitoxins, is equaled or surpassed by our lack of knowledge concerning the group of antibodies which are responsible for the agglutinin and precipitin reactions, or for the complement fixation and other immunological reactions, that is, the so-called specific immune amboceptors. As indicated previously, we are not even sure whether there are several such agents or whether all these reactions are accomplished by one sort of antibody. As far as chemical properties are concerned, we merely know that they are found in the globulin fraction of the serum, but we do not know whether the amboceptors are serum globulins modified by the process of immunization, or specific globulins formed in and secreted by the cells to unite with the antigen, or specific chemical radicals either attached to or forming part of the protein molecule. Manwaring has obtained evidence that the antibodies as existing and studied in serum may be different in character from the antibodies that exist in the whole blood, perhaps through changes that take place during the clotting.[61]

Landsteiner and Prasek [62] in 1911 reviewed the evidence advanced up to that time to demonstrate that antibodies are not proteins, and concluded that it was not competent to overthrow the weighty evidence establishing their close relation to the proteins. The evidence obtained by them through various procedures all supports the opinion that the antibodies are identical with the proteins which react with specific precipitins, and this conclusion seems still valid.[63]

[61] *J. Am. Med. Assoc.*, 91:386 (1928).
[62] *Z. Immunität.*, 10:68 (1911).
[63] However, Bleyer [*Z. Hyg.*, 108:302 (1928)] believes that he has found evidence that antibodies and the proteins of the immune sera are not identical, in view of their different resistance to sodium salts of alkylresorcinol carboxylic acids.

Isolation of Antibodies [64]

The chemical nature of the agglutinating and protective antibodies of antipneumococcus sera has been particularly studied by Huntoon [65] and his colleagues. Taking advantage of the possession of large amounts of this serum at the close of the war, they concentrated the antibodies by treating the serum with pneumococci, which unite specifically with the antibodies. After washing away the excess serum the antibody-antigen union is dissociated by extracting with weak alkaline solutions, and the bacterial substance removed by filtration through porcelain.[66] By this means solutions were obtained containing antibodies in a concentration equal to that of the best immune sera, and containing so little of the original serum protein that 5 cc. did not regularly sensitize guinea pigs to horse serum. Study of such purified preparations indicated that the antibody molecules are evidently colloidal or attached to colloids, as they do not pass through dialyzing membranes, but apparently they are not ordinary serum proteins since they resist trypsin digestion for long periods of time, do not give the usual protein color reactions, and are not precipitated by 30 per cent NaCl nor by removal of the electrolytes from their solutions; neither are they lipoidal in nature, since they do not dissolve in ether.

This probably represents as close as anyone has yet come to securing antibodies in approximately pure condition, but even here the amount of active substance present in the solutions available for study is so small that it is not possible to secure any satisfactory idea as to its chemical nature.

Huntoon's observations agree with somewhat similar studies made with hemolysins and hemagglutinins, concentrated by fixing the antibodies to red corpuscles, freeing the sensitized corpuscles from the serum, and then separating the antibody from the corpuscles, for the active solution of this antibody has been found to contain at the most but traces of recognizable protein (von Liebermann and Fenyvessy,[67] Kosaki [68]). In some of Huntoon's antibody solutions not only were all the conventional protein tests negative or doubtful, but although

[64] *See* review by Locke and Hirsch, in "The Newer Knowledge of Bacteriology and Immunology," Chapter LXXVIII. University of Chicago Press, 1928.

[65] Huntoon, Masucci and Hannum, *J. Am. Chem. Soc.*, 42:2654 (1920); *J. Immunol.*, 6:185 (1921).

[66] The antibodies may also be combined with the soluble specific carbohydrate substance of pneumococci, and then dissociated by acids and alkalies [Felton and Bailey, *J. Infect. Dis.*, 38:145 (1926)].

[67] *Centr. Bakt.*, 47:274 (1908).

[68] *J. Immunol.*, 3:109 (1918).

material precipitated by such reagents as picric, phosphotungstic and phosphomolybdic acid was present, the nitrogen content of the solution was as low as 0.16 mg. per cc.[69]

All these facts throw doubt on the protein nature of the antibodies, although they do not totally disprove it. On the other hand, Mudd's [70] studies of the behavior of the surfaces of bacteria and corpuscles before and after sensitization, support the view that the sensitization is accompanied by the addition of a surface film of protein, which is presumably the antibody. Furthermore, in contrast to Huntoon's results, Felton [71] found that the antibody of antipneumococcus serum is largely precipitated by simple dilution, thus resembling euglobulin, and denaturization of the protein precipitate by various means inactivates the antibody, as does digestive proteolysis. His further studies [71a] have indicated that the antibody activity of pneumococcus antiserum when in the most highly purified state obtainable, is associated with a water-insoluble protein which exhibits specific agglutinin, precipitin, complement-fixation, opsonic, and protective activities. Locke and Hirsch [72] also found that the purest hemolysins obtainable contain protein, from 0.000,125 to 0.000,18 mg. per unit, and believe that the failure of earlier workers to find proteins in their hemolysin preparations was due to lack of delicate methods for detecting proteins.

There is a striking similarity to be noted between antibodies, toxins, enzymes and insulin. Each of them acts in quantities that are so infinitesimally small that it is not possible to say whether the protein which may usually be found in the solution is itself the active agent or merely a contamination. Especially similar are the antibodies and the insulin, since each seems not to be the active agent itself, but merely one that accelerates or makes possible the action of other catalysts—that is, they are catalysts of the second order. Thus, the amboceptor serves to activate the complement; the insulin serves to make possible the burning of sugar by the tissues. Neither of itself accomplishes the final reactions. The amount of material necessary to accomplish this in the case of insulin is similar to the extremely minute figures obtained in quantitative studies of toxins, etc.

[69] Similar results have been obtained with typhoid antibodies by Uchida, *J. Infect. Dis.*, **40**:588, 597 (1927).
[70] *J. Exp. Med.*, **43**:127 (1926).
[71] *Bull. Johns Hopkins Hosp.*, **38**:33 (1926).
[71a] Felton, *J. Infect. Dis.*, **42**:248 (1928).
[72] *J. Infect. Dis.*, **37**:449 (1925).

The Site of Antibody Formation [73]

All attempts made to determine the place in the body where antibodies are formed, have so far led to no conclusive result. Such organs as can be excluded without destroying life have been removed from animals, without depriving them of the capacity to produce antibodies.[74] Since antibodies are constituents of the blood it has seemed reasonable to suggest that they are formed in the same place as many other of the elements of the blood, namely, the bone marrow and lymphoid structures. In support of this hypothesis are experiments in which it has been shown that stimulation of the blood-forming function of the bone marrow by such means as bleeding, leads also to a stimulation of the formation of antibodies, whereas serious injury to the marrow by x-rays or poisons,[75] such as benzene, reduces the capacity to produce antibodies.[76] Carrel [77] found that goat red corpuscles added to an artificial culture of guinea pig bone marrow and lymph gland stimulated the production of hemolysins in the culture. Przygode [78] also observed the formation of precipitins and agglutinins in cultures of spleen tissue, and Hektoen [75] has found that removal of the spleen often causes a marked reduction in the formation of antibodies. Addition of cholera vaccine to cultures of spleen tissue caused the production of demonstrable bacteriolysins (Schilf).[79]

However, there is not a little reason to believe that antibody formation may be a widespread cellular function, perhaps participated in by many or all the different kinds of cells in the body, for Fischer [80] found that even connective tissue cells in artificial culture may become immune to foreign sera. Local immunity may be greater in the tissues at the point where the immunizing antigen has been introduced than in other parts of the body.[81] There is also evidence that the fixed tissue phagocytes [82] or cells of the reticulo-endothelial system,[83] which are universally distributed, are especially active in the production of antibodies.[84] This is supported by the work of Portis,[85] who found that

[73] *See* review by Katharine Howell in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928.
[74] Hektoen, *J. Infect. Dis.*, **17**:409 (1915); **27**:23 (1920).
[75] Hektoen, *J. Infect. Dis.*, **17**:415 (1915); **22**:28 (1918); **26**:330 (1920); Simonds and Jones, *J. Med. Res.*, **33**:183 (1915).
[76] Hektoen, *J. Infect. Dis.*, **19**:69 (1916).
[77] Carrel and Ingebrigtsen, *J. Exp. Med.*, **15**:287 (1912).
[78] *Wien klin. Woch.*, **26**:841 (1913); **27**:201 (1914).
[79] *Centr. Bakt.*, **97**:219 (1926).
[80] *J. Exp. Med.*, **35**:661 (1922).
[81] *See* review by Hazen, *J. Immunol.*, **13**:171 (1927).
[82] Kyes, *J. Infect. Dis.*, **18**:277 (1916); Motohashi, *J. Med. Res.*, **43**:473 (1922); lit.
[83] Review by Maximow, *J. Infect. Dis.*, **34**:549 (1924).
[84] Billings, *Z. Immunität.*, **38**:193 (1923); Standenäth, *ibid.*, p. 19.
[85] *J. Infect. Dis.*, **34**:159 (1924).

the omentum of rabbits, which is rich in cells of this type, has much more capacity to produce antibodies against antigens injected into the peritoneal cavity, than the omentums of other animals which are not so abundantly provided with these cells. Tissue cultures of these same cells also show marked capacity to produce antibodies.[86]

Experiments performed by filling the phagocytic reticulo-endothelial cells throughout the body with foreign matter, such as india ink, iron, or colloidal dyes, indicate the importance of these cells in antibody formation, since in such "blockaded" animals the production of antibodies for injected antigens, is greatly reduced.[87]

The capacity of the body to produce antibodies is apparently but little affected by diets selected to prevent growth,[88] or deficient in accessory food factors, including vitamins [89] and various salts, except that possibly phosphorus deficiency reduces antibody formation (Zilva).[90]

Various inorganic salts injected into immunized animals may have a stimulating effect and serve to maintain the antibody content of the blood at a high level, according to Madsen.[91] This stimulating effect of salts is in support of the view that antibody formation is a true secretory process, but these results could not be confirmed by McIntosh and Kingsbury.[92]

The Coexistence of Antigen and Antibody in the Blood

Early in the study of immunity it was found by suitable experiments that during immunization there occur periods when both the antigen and its specific antibody apparently may be demonstrated in the serum of the immunized animal, evidently circulating together ununited. This unexpected condition, apparently paradoxical, aroused much interest and investigation. Three explanations have been suggested, according to Forster,[93] who reviews the literature as follows:

"Eisenberg in particular became sponsor for the suggestion that it could be explained by the chemical law of mass action, necessitating the belief that the two reacting substances existed partly in a free, partly in a combined state, equilibrium being maintained between them.

"Zinsser and Young, however, raise two objections to this explana-

[86] Meyer and Loewenthal, *Z. Immunität.*, **54**:409 (1928).
[87] *See* Siegmund, *Klin. Woch.*, 1:2307, 2566 (1922); Gay and Clark, *J. Am. Med. Assoc.*, **83**:1296 (1924); Vannuci, *Sperimentale*, **78**:23 (1924); Jungeblut and Berlot, *J. Exp. Med.*, **43**:613, 797 (1926); **44**:129 (1926).
[88] Hektoen, *J. Infect. Dis.*, **15**:279 (1914).
[89] Werkman, *J. Infect. Dis.*, **32**:247 (1923); **34**:447 (1924).
[90] *Biochem. J.*, **13**:172 (1919).
[91] *J. State Med.*, **31**:151 (1923).
[92] *Brit. J. Exp. Path.*, **5**:18 (1924).
[93] *J. Infect. Dis.*, **32**:105 (1923).

tion; first, that it has not been shown that colloidal reactions are governed by the law of mass action, the probability being that they are not; and, second, that the combined portion of the antigen-antibody complex should show a detectable complement-fixing power. Such they, as well as Gay and Rusk, have shown not to be the case. They seek an explanation in the close similarity between this phenomenon and other colloidal reactions, believing that other serum colloids exert a protective action, inhibiting precipitation."

Von Dungern [94] studied the same problem, coming to the conclusion that a multiplicity of antigens was responsible. Most of the recorded observations have been made with horse serum, and he points out that horse serum contains a variety of substances which act as antigens and produce corresponding antibodies. These antibodies are present in varying concentration in the serum of the immune animal and when horse serum is added to the antiserum one antibody may be present in excess so that it remains uncombined. Similarly, antigen may be present in excess of its antibody. The antigens and antibodies which coexist in the serum are not those which react with one another. According to his interpretation, therefore, while precipitinogen and precipitin actually may exist side by side without interaction, homologous precipitinogen and precipitin may not. As Forster says:

"Obviously the next logical step was the use of a single purified protein as an antigen. Weil employed crystallized albumin from the hen's egg as antigen and was unable therewith to detect the coexistence of antigen and antibody. This failed of confirmation, however, at the hands of Bayne-Jones, who, using crystallized egg albumin and edestin, carefully repeated Weil's work and demonstrated the coexistence of precipitinogen and precipitin with these pure substances. He also furnished additional support for the belief of Zinsser and Young in the protective action of certain serum colloids by showing that the presence of egg albumin in proper proportion could prevent the precipitation of a human serum by an antihuman serum."

Opie [95] repeated the experiments of Weil and Bayne-Jones with crystallized egg albumin, agreeing with the former and von Dungern that the presence of antigen and antibody in the supernatant fluid over a precipitate obtained by adding to antigen (horse serum, egg white, etc.) an amount of antibody in excess of that required to produce the maximum amount of precipitate, is best explained by the assumption

[94] *Centr. Bakt.*, Abt. I, 34:355 (1903).
[95] *J. Immunol.*, 8:19 and 55 (1923).

of a multiplicity of antigens in such complex mixtures as blood serum or egg white. The results obtained with crystalline egg albumin by Bayne-Jones he explains on the basis that, although this is an almost pure antigen, it contains in very small amount extraneous antigen capable of forming precipitin.

Studies of the coexistence of antigen and antibody in the living animal gave similar results, for Opie found that complex antigens, such as horse serum or egg white, injected into an immunized animal cause a diminution of the concentration of precipitins; but precipitins do not disappear completely, and in the early stages of immunization both antigen and antibody may be demonstrable in the same serum. A simple pure antigen, namely, crystalline egg albumin, purified by repeated crystallization, injected into an animal immunized against this substance may cause temporary but complete disappearance of precipitin, and though antigen may appear in the serum, in no instance were this antigen and its precipitin simultaneously demonstrable in the serum.

Crystalline egg albumin carefully prepared by the usual methods is a relatively pure antigen and brings about the formation of a single precipitin, and this precipitin cannot coexist with its specific antigen, except in the case of an excess of antigen producing the so-called "zone of inhibition," where precipitate is held in solution by excess of antigen so that antigen and antibody are side by side in the same fluid but do not combine to form a precipitate. This relation is found where antigen is in such excess that no precipitate is formed but exists in less degree whenever with increasing preponderance of antibody the latter is insufficient to form the maximum precipitate obtainable with a given quantity of antigen. In these mixtures the presence of antigen is readily demonstrated by addition of immune serum but, since the specific precipitate is already soluble in the excess of antigen in the mixture, addition of antigen will not result in the formation of a precipitate.

Opie [96] has also shown that the antibodies probably bind the antigen at the site of its introduction into the tissues, so that it ordinarily does not have an opportunity to coexist with the antibodies that are present in the blood of an immunized animal except when the injection is made directly into the vessels. If the antigen is injected into the subcutaneous tissues or the peritoneal cavity of a well-immunized animal, its entrance into the blood cannot be demonstrated at all unless overwhelming doses are employed. On the other hand, if proper amounts of antigen are

[96] *J. Exp. Med.*, 39:659 (1924).

injected into the blood of an immunized animal the antibodies may be made to disappear from the blood.

From all the above facts it would seem that the supposed coexistence of antigen and antibody in the blood is not an actual occurrence, but the result of inadequate methods of investigation. This chapter in immunological history serves to emphasize the principle that the *fundamental processes of immunity should be studied only with simple antigens of known composition, i.e., isolated pure proteins.*

Recapitulation

The chemical nature of antibodies is entirely unknown, for as yet they have not been isolated certainly free from other substances, and the purest preparations so far obtained contain but infinitesimal amounts of the active material. Like the enzymes, they are recognized by what they do, rather than by what they are.

Their existence is recognized by the numerous different reactions they accomplish, to which descriptive titles are given, such as agglutinin, precipitin, complement fixation reactions, etc. But it is not known whether each of these reactions is produced by a definite and individual sort of antibody (e.g., agglutinin, precipitin, amboceptor), or whether a single antibody accomplishes all these reactions which may differ merely in the method used for their demonstration.

There is much evidence in favor of the view that there are two classes of antibodies. One, the antitoxins, possesses the power to neutralize toxic antigens. The other is characterized by so acting upon foreign proteins that their colloidal state is altered, either by aggregation of the colloids (agglutination, precipitation) or by dispersion (lysis), in the latter case the antibody merely preparing the antigen for subsequent action of the lytic agent. A highly purified antibody preparation has been found to exhibit all these different sorts of antibody reactions. Presumably all the different reactions of the second group depend on a single sort of antibody, and it is even possible that this is the same as the antitoxins. It is certainly true that the injection of a single antigen, even if this be a purified protein, leads to the presence in the serum of the immunized animal of antibody activity by which several or all of the known immunological reactions can be demonstrated, and it is much easier to believe that the single pure antigen incites the formation of one rather than of many distinct antibodies. Plausible as this hypothesis is, however, there are some observations not agreeing with it which have not been explained away to the

satisfaction of everybody; hence it cannot now be said to be fully established.

Although antibodies have not been isolated it is generally held that they are proteins, and antibody activity is usually destroyed by agents that alter the character of serum proteins, while inactivation generally parallels denaturation of the proteins. They are found associated with the globulin fraction of the serum, antitoxins being separated out in the pseudoglobulins, although some other antibody reactions are exhibited more with the euglobulins. Nevertheless, it is not yet certainly established that antibodies are proteins. Some of the purest and most active antibody preparations that have been made have held so little demonstrable protein that there has been much reason to doubt that the antibodies could themselves be proteins; nevertheless, even these purest preparations behave as if of colloidal character. It is possible that the antibody character resides in relatively small groups or radicals which can function only when attached to some colloidal complex of large molecular dimensions. In favor of the view that antibodies are associated with definite proteins is the fact that in separating antibodies from serum they come down quite constantly in certain fractions and not in others which may be separated first from the serum without carrying along any of the antibody activity.

Apparently antibodies are formed in many parts of the body, but just how, by what cells and by what processes, will probably remain unknown until they have been more accurately identified. There is much reason to believe that the endothelial cells throughout the body, especially that type active in phagocytosis and often designated as reticulo-endothelium, may be particularly concerned in antibody formation.

CHAPTER V

THE NEUTRALIZATION OF TOXIN BY ANTITOXIN

As indicated in the preceding chapter, the toxin-antitoxin reaction seems to be somewhat different from the other immunological reactions which have certain common features indicating their close relationship to one another, if not an identity. The difference lies in the fact that the antitoxin inhibits directly the poisonous properties of a specific toxin, whereas with the other reactions no such detoxicating effect is necessarily accomplished, for the antigen is commonly not toxic, and often, indeed, the lytic reactions lead to the production of injurious effects by non-toxic antigens.

Definition of Toxins

It will be recalled that in immunology the use of the term toxin is limited to that group of substances which are primarily toxic and which produce immunity in animals through inciting the formation of specific antibodies, the antitoxins, which neutralize the toxins *in vitro* and *in vivo*. Under this generally accepted definition, toxic substances which do not engender antitoxin formation are not toxins (e.g., alkaloids), and antibodies for non-toxic substances are not antitoxins (e.g., antibodies for non-toxic foreign proteins). Certain toxic foreign proteins, e.g., ricin, may cause the production of specific antitoxins, and hence are considered to be toxins, while toxic bacterial products which do not incite production of specific antitoxins (e.g., ptomaines, "endotoxins") are not classed with the toxins. On the basis of this definition of the term toxin the number of true toxins so far recognized is small, including only the bacterial toxins of diphtheria, tetanus, scarlatina, gas gangrene, botulinus, pyocyaneus, symptomatic anthrax, and perhaps poisons of minor importance produced by *B. dysenteriæ, B. typhosus, B. paratyphosus,* various strains of streptococci, and the cholera spirillum; also certain bacterial hemolytic poisons (hematotoxins), and leucocyte-destroying poisons (leucocidins); a small group of highly active toxins of vegetable origin, the phytotoxins, namely, ricin, crotin, robin, abrin and curcin; and certain poisons of animal origin including

snake venom, the poisons of scorpions, spiders, and eel serum. Poisonous bacterial antigens which do not incite the production of antitoxins are commonly called "endotoxins," in contrast to the soluble, excreted, antitoxin-producing "exotoxins."

The Nature of the Toxin-Antitoxin Reaction

The neutralization of a toxin by its specific antitoxin was at one time believed by many investigators to be a simple chemical process, which occurs as well in the test-tube as in the body. It was believed to occur according to the laws of definite proportion, a given amount of antitoxin neutralizing a proportionate amount of toxin under equal conditions. Later investigations have shown that the reaction is not so simple a process, and the views now prevailing will be discussed in the following pages. One important feature of the toxin-antitoxin reaction is that neither the toxin nor the antitoxin is destroyed in the process of neutralization, as has been proved by suitable experiments, but they appear to be united to each other, as any two large molecules may be, whether chemically or by physical adsorption. Thus, if a mixture of snake venom neutralized with antivenin is heated at 68° for some time, the more thermolabile antitoxin is destroyed and the toxicity of the venom is restored (Calmette).[1] Similar results have been obtained with other toxin-antitoxin mixtures. Apparently the union of toxin and antitoxin requires some time, for Martin and Cherry [2] found that when the neutral venom-antivenin mixture is filtered through gelatin filters under pressure, the free toxin molecules alone pass through, and the longer the mixture has stood before filtration the less toxin can be recovered.

Eisler [3] found that if a precipitating serum for horse serum is added to a neutral mixture of tetanus toxin and antitoxin which has not stood over one-half hour, the activity of the toxin is restored by the union of the horse serum proteins with precipitin, indicating that the antitoxin is a specific serum protein not strongly united to the toxin in this space of time; but 50 per cent ammonium sulfate, which precipitates free antitoxin, does not set free the toxin from its union with antitoxin. Even toxin-antitoxin compounds that are so firmly bound after longer standing that they are not separated by the action of precipitins, nevertheless are somewhat modified thereby, for they become much more readily separable by acids.

[1] *Ann. Inst. Pasteur*, 8:275 (1894).
[2] *Proc. Royal Soc.*, 63:420 (1898).
[3] *Centr. Bakt.*, 84:46 (1920).

There is some question as to whether the union with antitoxin completes the neutralization of the toxin, or whether there is then necessary a further destruction of the toxin in the body. *In vitro* no such destruction seems to take place even when the toxin and antitoxin are together for long periods of time. Neutralization occurs more rapidly under the influence of warmth, and more slowly in the cold; and it is more rapid in concentrated than in dilute solutions, just as with ordinary chemical reactions. It is said that it requires two hours for tetanus toxin to be completely combined with the corresponding quantity of antitoxin at 37°.

In general, the union of toxin and antitoxin can be dissociated by acids.[4] Even after standing as long as three months a neutral toxin-antitoxin mixture may be dissociated by HCl and the presence of active toxin and antitoxin demonstrated by suitable methods,[5] showing conclusively that the neutralization is not accompanied by any marked alterations in either component.[6] On dilution of a neutral toxin-antitoxin mixture, a certain amount of dissociation occurs, but not sufficient to support the view that the law of mass action applies to the reaction between toxin and antitoxin. Such mixtures seem to dissociate gradually after injection into the animal body, this being the basis for the production of active immunity by injection of toxin-antitoxin.[7]

Toxin-antitoxin Precipitates

Nicolle[8] and others have described the production of a precipitate when toxin is neutralized by antitoxin, and this has been developed by Ramon as a means for assaying the strength of antitoxins. It has been suggested that the precipitate results from a reaction between proteins present in the bacterial filtrate and precipitins for these proteins present in the antitoxic serum,[9] rather than between toxin and antitoxin, but the fact that precipitins for bacterial proteins do not precipitate toxins from

[4] Morgenroth and Ascher, *Cent. Bakt.*, **59**:510 (1911).

[5] De Potter, *Bull acad. roy. med. Belg.*, **3**:394 (1923).

[6] The union of diphtheria toxin and antitoxin may be dissociated to some extent by freezing in the presence of phenol or tricresol so that intoxications have resulted from injection of frozen toxin-antitoxin mixtures used for immunizing children against diphtheria. [Anderson and Leonard, *J. Amer. Med. Assoc.*, **82**:1679 (1924).] Such dissociation is less likely to occur if the antitoxin has been purified before its union with the toxin.

[7] *See* Schönberger, *Z. Kinderheilk.*, **41**:546 (1926).

[8] Nicolle, Debains and Césari, *Compt. rend. Acad. Sci.*, **169**:1433 (1919); *Ann. Inst. Pasteur*, **34**:709 (1920); Ramon, *ibid.*, **37**:1001 (1920); **38**:1 (1924). Review by Bayne-Jones in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, p. 759. 1928.

[9] Georgi [*Med. Klinik*, **16**:1053 (1920)] has found that when toxin and antitoxin are brought together with addition of lipoidal extracts as in the Wassermann reaction, there occurs a flocculation of material which Niederhoff found to consist of lipoids,

the same bacteria (Schmidt, Bayne-Jones [8]) is not in favor of this view.[9a]

The precipitates of diphtheria toxin and antitoxin prepared by the method of Ramon,[10] have been found to consist chiefly of alcohol-insoluble protein, 73.4 per cent; with alcohol-soluble protein, 8.1 per cent, and lipin 18.5 per cent [11] after subtracting inorganic salts. Hartley [12] found that no precipitate forms if the toxin and antitoxin have previously been freed from lipoids, although neutralization occurs, and therefore suggests that the flocculation is of lipoids which carry down the toxin-antitoxin compound.[13] From such floccules the antitoxin may be separated in high concentration (Locke and Main). Bayne-Jones finds that the evidence as accumulating is in favor of the view that it is truly a specific toxin-antitoxin flocculation reaction,[14] and not, as has been suggested, solely a precipitation of bacterial proteins independent of a toxin-antitoxin reaction.[15] Apparently the floccules of precipitate are not of constant composition, but seem to contain more toxin or more antitoxin according to which of these is in excess in the original mixture.[16]

Physical Chemistry of the Reaction

According to Arrhenius and Madsden, reaction of antitoxin upon toxin is accompanied by the liberation of much heat—6600 calories per gram molecule, or about half as much as is set free by the action of a strong acid upon a strong base.[17] Union of toxin and antitoxin causes no change in the surface tension of the fluid in which the reaction occurs (Zunz),[18] and the neutral toxin-antitoxin compound (diphtheria) is not adsorbed by animal charcoal, which adsorbs each of the constituents when free. The physico-chemical studies of the reaction between tetanolysin and its antibody gave results which led Arrhenius to conclude that in the reaction there are formed from one molecule of toxin and one molecule of antitoxin, two molecules of the reaction

[9a] *See also* Hoen *et al.*, *Z. Hyg.*, **108**:61 (1927).
[10] *Ann. Inst. Pasteur*, **37**:1001 (1923).
[11] *See* Locke and Main, *J. Infect. Dis.*, **39**:484 (1926).
[12] *Brit. J. Exp. Path.*, **6**:180 (1925).
[13] *See also* Friedberger and Ikeda, *Klin. Woch.*, **4**:2149 (1925).
[14] The decrease in residual nitrogen observed by Fuchs and Falkenhausen [*Biochem. Z.*, **176**:92 (1926)] to occur when toxin and antitoxin are mixed, is considered by them to indicate that the toxin-antitoxin union is chemical. This change in nitrogen distribution might, however, depend on many other possible occurrences.
[15] Bronfenbrenner and Reichert, *J. Exp. Med.*, **44**:553 (1926).
[16] *See* Hartley, *Brit. J. Exp. Path.*, **7**:55 (1926).
[17] Literature of chemical and physical reactions of toxin and antitoxin given by Zangger, *Centr. Bakt.* (*ref.*), **36**:238 (1905); Arrhenius, "Immuno-chemistry," 1907, and "Quantitative Laws in Biological Chemistry," London, 1915; also review in *Z. Chemother, Ref.*, **3**:157 (1914); Oppenheimer and Michaelis, "Handbuch der Biochemie," Vol. II (1).
[18] *Bull. Acad. Royal Med. Belg.*, **25**:425 (1911); also Bertoline, *Biochem. Z.*, **28**:60 (1910).

products, analogous to the reaction between alcohol and acid which yields one molecule of ester and one of water. It must be recognized, however, that in all attempts to study the physico-chemical characters of toxin-antitoxin reactions, there lies a fundamental difficulty in that the reactions under observation were never those of pure antitoxin with pure toxin, but of a most complex mass of bacterial products in a complex broth mixture, reacting with an antiserum which contained an unknown number of substances. How much of the observed phenomena depended on union of toxin and antitoxin, and how much on reactions between the innumerable other substances present, it is not possible to know, and the value of all the data obtained with such materials is doubtful, to say the least.

Relation to Enzyme Action

There seems to be no close resemblance between the method of action of antitoxins and of enzymes. The antitoxin acts quantitatively, and produces no detectable alteration in the toxin, or in any other substance, as far as we know. In the Ehrlich nomenclature, it has but one functioning group (haptophore), the one with which it combines with the toxin; whereas both toxins and enzymes seem to have two functioning groups, one which unites with the cell or substance that is to be attacked, the other which produces the chemical changes. But there is evidence that union with antitoxin or fixed receptors prepares the toxin for its subsequent disintegration within the body, which, presumably, is then accomplished by enzymatic action as in the destruction of other antigens.

However, Zinsser [19] points out the similarity of antigen-antibody reaction curves to some of the effects seen in the action of enzyme on its substrate. Thus, Northrop found that the apparent divergence of pepsin action from the results predicted from the law of mass action, can be quantitatively explained by the fact that the enzyme in solution combines with some of the substances produced by the digestion, and maintains an equilibrium with these substances which obeys the ordinary laws of mass action. As the pepsin digests the protein, peptone-like substances are formed with which pepsin goes into combination and with which it then maintains an equilibrium following mass action laws. Since it is only the uncombined or dissociated pepsin which affects the further hydrolysis, the curves experimentally obtained may be readily explained. In adding increasing amounts of peptone to pepsin solution,

[19] "Infection and Resistance," 3rd Edition, p. 142.

as a result of this manner of combination, the first amounts added inactivate more pepsin than the later additions. As Northrop points out, this is in very striking analogy to the manner in which antitoxin and toxin react in Ehrlich's experiments. Similar observations were made with tryptic digestion. As Zinsser says: "These experiments of Northrop do not, of course, solve the question of antigen-antibody unions, but they do serve to bring the analogy of toxin-antitoxin relations much closer to laws governing the union of enzyme with substrate. Moreover, they show that enzyme is actually used up in its reactions, just as toxin is used up in its reactions with antitoxin, and that equilibrium following the laws of mass action may be a definite factor in the quantitative relations governing the reactions."

Ehrlich's Theory [20] of Toxin-Antitoxin Neutralization

Ehrlich and his colleagues, finding that the neutralization of toxin by antitoxin was not so simple a process as had at first appeared, developed a very elaborate and complex theory as to the factors involved. They determined four different values as standards or units for the measurement of diphtheria toxin-antitoxin neutralization, namely:

(1) M. L. D., the minimum lethal dose, (or T), for a 250 gm. guinea pig in four days.

(2) Antitoxin unit = amount of antitoxin which neutralized 100 M. L. D. of a certain diphtheria toxin.

(3) L_0 = Limes or threshold dose, i.e., the amount of toxin which is just neutralized by one antitoxin unit.

(4) L_+ = the minimum amount of toxin which will cause death in four to five days if injected together with one antitoxin unit into a standard guinea pig.

It was soon found that a toxin solution rapidly loses its toxicity on storage, without a corresponding loss in its capacity to neutralize antitoxin. This led to the view that the poisonous element of the toxin must be different from the constituent which unites with the antitoxin, from which arose the conception that the toxin consists of two elements, a toxic or "toxophore" group, and a binding or "haptophore" group. When, through the action of time or certain chemical or physical agents, the toxophore group is thus impaired or removed, the remaining nontoxic group still possesses the power of binding antitoxin or of immunizing animals against the toxin, and Ehrlich called this a "toxoid."

[20] For critique of Ehrlich's theories *see* Manwaring in Chap. 81, "The Newer Knowledge of Bacteriology and Immunology." Univ. of Chicago Press, 1928.

Another discrepancy appeared in the measurement of the L_+ dose. It would be expected that this must be just one M. L. D. more than the L_0 dose, but as a matter of fact it requires many additional M. L. Doses to kill a guinea pig with a toxin-antitoxin mixture after completely neutralizing one antitoxic unit. This observation made it necessary to assume that there are different degrees of affinity for antitoxin in the toxoids present in the toxin solution. Toxoids with a weaker affinity for antitoxin than toxins were designated as "epitoxoids" and it was also assumed that there exist other substances, "toxons," which are formed as such by the diphtheria bacillus, having the same sort of haptophore group as the toxins, but a different toxophore which gives rise, not to acute symptoms but to slow emaciation and paralysis.

Another development was the appearance of phenomena in the course of quantitative studies of toxin-antitoxin neutralization which made it necessary to assume that there must be other toxoids which have a higher affinity for antitoxin than the toxin itself, and these were christened "protoxoids." Also there seemed to be toxoids with the same affinity for antitoxin as the toxins, and they were given the name "syntoxoids." In order to visualize the action of such a complex mixture as the toxic filtrate of diphtheria cultures had shown itself to be, Ehrlich described a "spectrum" of reactions occurring between the filtrate and antitoxin.[21]

The toxic filtrate from diphtheria bacillus cultures was supposed to contain the following ingredients reacting with the antitoxin:

A. Toxins and toxons, differing in the acuteness of their toxic effects, both produced as such by the diphtheria bacillus and both uniting with the antitoxin.

B. Toxoids, derived by disintegration of the toxins and perhaps also the toxons, and of varying degrees of affinity for antitoxin, e.g., protoxoids, syntoxoids.

C. Other possible modifications, for apparently each of these subdivisions of the toxins consist of equal parts of two components which have similar affinities for antitoxin but different capacities to resist deterioration.

These highly complex conditions were assumed by Ehrlich on the basis of the conception that toxin-antitoxin reactions are entirely analogous to those occurring between strong acids and strong bases, in which the reactions are complete in one direction and reversible only to an inappreciable degree — e.g., $H_2SO_4 + 2NaOH = Na_2SO_4 + H_2O$.

[21] *Deut. med. Woch.*, 24:597 (1898).

Arrhenius' Critique of the Ehrlich Theory.

Arrhenius and Madsen,[22] however, suggested that the toxin-antitoxin reaction might be more similar to that between weak acids and bases, in which the equilibrium is reached while there are present appreciable amounts of the free acid and base as well as of the neutral salt, the quantities depending, according to the law of Mass Action, upon the relative proportions in which the two reagents are present in the reacting mixture. These investigators took for their chemical comparison the reaction between boric acid and ammonia, because as ammonia is hemolytic and boric acid is not, the amount of hemolysis produced by such a mixture gives an indication of how much free ammonia there is in the mixture. Because of the reversibility of the reaction between ammonia and boric acid there is always present some free ammonia even when boric acid is added to saturation. They compared the behavior of such hemolytic mixtures of ammonia and boric acid with the neutralization of hemolytic toxins by the specific antitoxin, and found a marked similarity in their behavior, and also, a behavior similar to that observed by Ehrlich in the neutralization of diphtheria toxin by antitoxin. That is, if boric acid is added to a solution of ammonia the hemolytic power of the solution is reduced by the first portions of acid, to a degree directly proportional to the amount of acid added, but later additions of acid have each less and less effect upon the hemolytic power of the solution until a point is reached where no further appreciable neutralizing effect is obtained on adding more boric acid, a small residual hemolytic action being manifested by the ammonia which is always present through hydrolysis of the ammonium borate.

As shown in the diagrams on page 140, the curve of neutralization of the hemolytic poison produced by the tetanus bacillus (tetanolysin) by its antitoxin, is similar to that of the neutralization of the hemolytic action of ammonia by boric acid.

They therefore believed that the phenomena observed by Ehrlich in the toxin-antitoxin neutralization can also be explained on the same physico-chemical basis, as depending upon the presence of varying quantities of free toxin dissociated from a salt-like toxin-antitoxin compound, rather than upon the highly artificial structure of toxins, toxoids and toxons imagined by Ehrlich. To quote Arrhenius:[23] "This behavior is to a high degree similar to that termed Ehrlich's phenomenon, observed in the neutralization of a toxin with its antitoxin. The

[22] "Immunochemistry," Macmillan, 1907.
[23] "Immunochemistry," p. 177.

first part of the antitoxin added neutralizes, generally speaking, a greater portion of the toxin than does the second equal addition, this

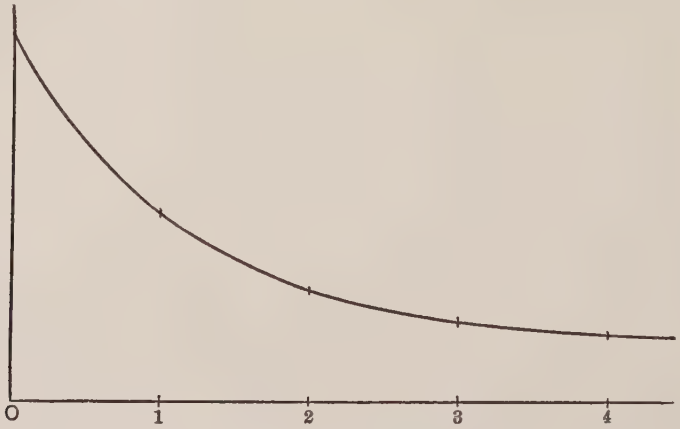


FIG. 1.—Curve of hemolysis by ammonia during progressive neutralization by addition of constant quantities of boric acid.

a greater one than the third, and so forth. To explain this peculiarity (of diphtheria toxin) Ehrlich supposes that the toxin is a mixture of

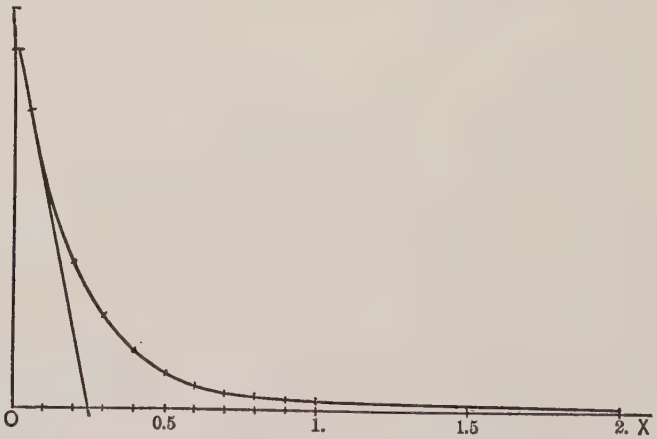


FIG. 2.—Curve of hemolysis by tetanolysin during progressive neutralization by antitoxin.

many different 'partial toxins,' which possess different degrees of toxicity in equivalent quantities, and have a different affinity for antitoxin. If antitoxin be added, it at first neutralizes that part of the poison which has the greatest affinity, and which also is the strongest

poison; thereafter that with the next greatest affinity, which also is the second in toxic strength, and so forth. At the end the very weakest portions appear for neutralization. Ehrlich designated these hypothetical 'partial poisons' with names coined from the Greek language, as protoxin, deuterotoxin, tritotoxin, epitoxin, etc.

"If we apply Ehrlich's views to ammonia, this substance should, according to the experiment of neutralization by boric acid, be composed of different 'partial ammonias,' of which the strongest one should be neutralized first, the second strongest next, etc. Of course this complicated explanation cannot possibly be used for ammonia, which we know is a very simple chemical compound of high purity, but it was by Ehrlich and his pupils applied to other poisons quite generally, e.g., to diphtheria-poison and tetanolysin, which, on neutralization, as we shall soon see, behave in a manner very similar to ammonia."

Attractive as is this hypothesis of the chemical nature of antigen-antibody reactions, which assumes them to be simple salt-like unions establishing an equilibrium according to the laws of mass action, it has not been found acceptable as a general principle to explain the union of antigens and antibodies, or even for the specific case of toxin-antitoxin neutralization. Simple dilution of such a neutral mixture does not accomplish the amount of dissociation of toxin and antitoxin that this theory calls for. Furthermore, as Grassberger and Schattenfroh [24] learned in their studies of symptomatic anthrax toxin and antitoxin, dilution renders neutralization much more difficult and different mixtures are obtained depending on whether they mix the toxin and the antitoxin after diluting them, or dilute the toxin-antitoxin mixture. This fact is evidently not in favor of the Arrhenius-Madsen theory, according to which the same state of equilibrium should exist in both instances owing to reversibility, and the same fraction of the toxin of necessity remain free. Again, the *Danysz phenomenon* (see p. 143), which is typically exhibited by toxin-antitoxin mixtures, is not generally considered as compatible with this straight chemical theory, although Arrhenius [25] does find some analogies in known chemical reactions.

Attempts to observe changes in the refraction of mixtures in which antigen-antibody reactions are taking place, have given negative results,[26] which fact also speaks in favor of the physical rather than the chemical nature of these immunity reactions, which, unfortunately, did

[24] Quoted from Bordet, "Studies in Immunity," Bordet-Gay, p. 521, 1909.
[25] *Z. Chemother., Ref.*, 3:420-429 (1914).
[26] Doerr and Berger, *Biochem. Z.*, 123:144 (1921); Bachmann, *Z. Immunität.*, 35:462 (1923).

not include toxin-antitoxin neutralization.[27] However, Zunz found no change in the surface tension to result from the union of toxin and antitoxin.

The Adsorption Theory of Bordet [28]

Bordet, who from the first has urged that immunological reactions are fundamentally physico-chemical, explains the spectrum phenomena of Ehrlich on the basis of adsorptions. That is, he does not imagine that when one combining unit of antitoxin is added to ten units of toxin, one of the latter unites quantitatively with the antitoxin unit, leaving nine units of completely unaltered toxin still present in the solution as in a simple chemical reaction. Rather, the one unit of antitoxin is distributed upon all the toxin molecules present, modifying somewhat but not completely neutralizing their toxicity.[29] Thus, Ehrlich's "toxons" become merely incompletely neutralized toxin, although the existence of spontaneously detoxicated toxin molecules in the form of toxoids is not denied. For the theory of an exact union of toxin with antitoxin in fixed definite proportions like a simple chemical reaction, Bordet substitutes the hypothesis of union in variable proportions according to concentration and other varying factors obtaining in the solutions in which the reaction takes place. Such a conception is much more closely in harmony with reactions occurring in colloidal systems, which toxins and antitoxins are. Bordet compared such reaction to the phenomenon familiar in dyeing, for the dye colors all parts of the substance, faintly or deeply according to the proportion of dye and adsorbent.

When tested in the laboratory, Bordet's "Adsorption Theory" receives much support. For example, bacteria, corpuscles, or tissue cells in a given supension with immune serum present, can be shown to be all sensitized in varying degrees according to the amount of available antibody present. The fact that antigen-antibody unions do not undergo any considerable dissociation on dilution, and that after such unions have stood for a few hours dissociation is usually too little to be detected and the union becomes increasingly firmer with longer standing, is not in harmony with the idea of Arrhenius and Madsen that such unions are dissociable compounds analogous to the salts of weak acids and bases. In this respect their behavior is closely analogous to that of

[27] Vles [*Compt. rend. Acad. Sci.*, 171:524, 552 (1920)] has stated that tetanus toxin, when examined spectrophotometrically with violet light, gives three absorption bands, varying in intensity directly with the toxicity. When inactivated either by heat or antitoxin, these bands are similarly removed.

[28] For full review *see* "Studies in Immunity" by Jules Bordet and F. P. Gay, Wiley and Sons, 1909.

[29] *Ann. Inst. Pasteur*, 17:161 (1903).

colloidal unions, e.g., (1) dyeing with colloidal dyes, which attach themselves progressively more closely to the substrate with a correspondingly decreased tendency to be washed out; (2) the increasing insolubility of proteins precipitated by and standing in alcohol.

Field and Teague [30] found that both toxin and antitoxin in either neutral or alkaline solution move toward the cathode, and pointed out that this was against the assumption of a neutralization of two oppositely charged molecules, and in favor of the purely colloidal nature of toxin-antitoxin neutralization. Reinvestigation by Maver and Falk [31] showed that both toxin and antitoxin behave as amphoteric colloids. Bechhold [32] pointed out the fact that chemical combination may occur between two substances of the same reaction, and that the assumption of a pure physico-chemical colloidal reaction *fails to account for specificity*. He called attention to the similarity of the reactions in the carbohydrates, which are so much affected by even stereo-isomeric alterations in the molecule.

Nevertheless, many well-known phenomena of immunology other than toxin-antitoxin neutralization also harmonize best with the view of Bordet that these reactions are, at least in their first steps, adsorption reactions between colloids, and they will be discussed in the consideration of the several types of immunological reactions on other pages. Among these phenomena may be mentioned here the zone reactions and the Danysz effect.[33]

By the **"zone phenomenon"** which is discussed more fully on p. 177 is meant essentially that only with proper proportions of immune bodies and antigens can optimum effects be produced, excess of any one of the reacting bodies beyond certain limits reducing the effect. Thus a precipitating serum which gives strong precipitin reactions with the specific antigen when diluted 1-10,000 or even much more, may show no precipitation whatever in concentrations of 1-100 or greater; a bactericidal serum in a high dilution may kill bacteria and fail to do so in a stronger concentration, and so on. Such behavior does not fit well with any hypothesis of the antigen-antibody reactions as simple chemical reactions, whether one follows the ideas of either the Ehrlich or the Arrhenius school, but does correspond with well-known facts concerning colloidal reactions; e.g., when two colloids of opposite electrical charges are mixed in suitable proportions they may be precipitated, but an excess of either one may partially or entirely prevent the precipitation or redissolve a precipitate that has been formed.

The **"Danysz effect"** is the term applied to the observation that when an excess of toxin is added to its specific antitoxin in several portions at proper intervals of time, there is left much more unneutralized toxin in the mixture than if the

[30] *J. Exper. Med.*, 9:86 (1907).
[31] *J. Immunol.*, 14:219 (1927).
[32] *Münch. med. Woch.*, 54:1921 (1907).
[33] *Ann. Inst. Pasteur*, 16:331 (1902).

same quantity of toxin had been added to the same quantity of antitoxin at one time. The same phenomenon was described for hemolysins in 1900 by Bordet. Such a behavior is identical with that seen in colloidal reactions, e.g., if a piece of blotting paper is torn into pieces which are dropped into a dye solution one at a time, they will take up more of the dye than will the same amount of blotting paper added in one piece.

Up to a certain point, at least, the colloidal adsorption theory of Bordet fits better with the known facts than the definite chemical theories of Ehrlich and Arrhenius, and has received more and more attention and support since the days when the brilliancy and aggressive presentation of Ehrlich's conceptions overwhelmed immunological investigators. Nevertheless it has to be recognized that the status of colloidal reactions is at present too unsettled to permit of final interpretation of their application to immunological processes, and especially so since the studies of Jacques Loeb have emphasized the possibility that the so-called colloidal behavior of proteins is dependent on purely chemical processes not essentially altered by the colloidal state of the proteins.

The chief shortcoming of the colloidal adsorption theory of immunological reactions is its failure to explain adequately the *specificity* of the reactions, which, as pointed out elsewhere (Chapter III), seem to depend chiefly on definite chemical differences in the antigens. To account for both the harmonies and shortcomings of the purely colloidal explanation of the toxin-antitoxin reaction advanced by Bordet, and supported by Biltz and others, various investigators have suggested its amplification by assuming a primary colloidal adsorption and a secondary specific chemical reaction, as Pick and Schwartz have done. Theoretically, adsorption processes might be highly specific, but the fact is that as yet nothing corresponding to immunological specificity has been found in colloid reactions of non-biological character.[34]

Von Krogh [35] has discussed at length the attempts which have been made to explain the toxin-antitoxin reaction as an adsorption, and concludes that such a purely physical process explains neither the specificity of the reaction nor the neutralization. He found that although toxin is adsorbed by any such positive colloid as iron hydroxide, strictly according to the adsorption formula, yet it remains quite as toxic as before. In favor of the physical nature of the toxin-antitoxin reaction as a colloidal adsorption process, Krogh cites the firmness of the fixation, its lack of reversibility, its union in successive steps, and the

[34] *See* Freundlich, "Colloid and Capillary Chemistry," 1922.
[35] *J. Infect. Dis.*, 19:452 (1916).

Danysz phenomenon, all of which features are characteristic of adsorptions and harmonize with the colloidal character of both toxin and antitoxin. But as the adsorption of the toxin by a colloid other than antitoxin does not neutralize the toxin, it would seem that the reaction is something more than a purely physical adsorption, possibly a chemical reaction within the colloidal complex. He is, therefore, in agreement with Pick and Schwartz and many other students of immunology, who seem inclined to accept some such compromise view of the matter.[36]

Recapitulation

The toxin-antitoxin reaction is characterized by the fact that here a specific chemical poison is neutralized by a specific antibody, without the destruction of either, for neutral toxin-antitoxin mixtures may be dissociated by various means into the active components. Ehrlich developed an elaborate theory of the processes and agents involved in this reaction, which he assumed to resemble in principle the neutralization of a strong acid by a strong base. To fit this theory to the many observed facts it became necessary to imagine the toxin as composed of many components of varying degrees of toxicity and with varying affinities for antitoxin, and the theory finally became so complex as to collapse under its own weight.

Arrhenius and Madsen studied the physical chemistry of toxin-antitoxin neutralization and advanced the hypothesis that it resembled more the reaction between weak acids and bases, in which equilibrium occurs according to the law of mass action. As an illustration they presented the neutralization of ammonia by boric acid, which leads to an equilibrium in which there is always present free ammonia even when boric acid is added to saturation. The hemolytic action of ammonia in such a neutralization mixture was found to parallel closely the neutralization of hemolytic toxins by the specific antitoxins.

Bordet looks upon the toxin-antitoxin neutralization as more like the neutralization of two colloids of different charges, and advanced an "adsorption theory." He does not accept the simple chemical explanation that toxin and antitoxin unite in fixed definite proportions, but believes that the process is more similar to the dyeing of a colloidal fabric by a colloidal dye. There seems to be a growing body of evidence in support of this theory as explaining best many of the observed

[36] Traube [*Z. Immunität.*, 9:246 (1911)] advanced a "resonance theory" which assumes that the specificity is determined by a "tuning" (Abstimmung) of the surface forces of antigen and antibody, but, attractive enough as a pure hypothesis, it lacks up to the present time any experimental demonstration.

phenomena of toxin neutralization, as well as other immunological reactions. For example, such typically colloidal phenomena as the zone phenomenon of inhibition or reversal of reaction by an excess of one agent, and the "Danysz effect" of unlike results produced by the same quantity of agent when added in single or in fractional doses, are exhibited in toxin-antitoxin neutralization.

However, the simple colloidal adsorption theory, as so far developed, fails to account for the specificity of the immunological reaction. Consequently, there is a tendency at present to look upon adsorption as only one part of the toxin-antitoxin neutralization, perhaps the first step that brings together the antigen and antibody, secondary chemical processes occurring in the colloidal complex accounting for the specificity of the reaction and the neutralization of the toxin.

CHAPTER VI

AGGLUTINATION AND PRECIPITATION

As pointed out in the preceding chapter, there is every reason to believe that these two immunological reactions depend on identical agents and represent the same processes, differing only in that they are carried out with particulate protein matter (cells) in the case of agglutination and with dissolved protein in the case of precipitation. For example, if we immunize with whole typhoid bacilli the immune serum will equally well agglutinate typhoid bacilli and give precipitin reactions with a filtered extract of typhoid organisms which contains the dissolved bacillary proteins. Or if we immunize with an extract of typhoid bacilli the antiserum will agglutinate typhoid bacilli and give precipitin reactions with the extract. Indeed, if colon bacilli are suspended in a solution containing soluble typhoid substance, they will adsorb enough of the typhoid antigen so that they will be agglutinated by antityphoid serum (Arkwright).[1] Again, if bacteria or minute collodion particles are exposed to a protein in solution, then washed as free as possible from unadsorbed protein, they can be agglutinated by a serum which contains specific precipitins for this protein (Jones).[2]

Therefore it is appropriate to consider these two reactions together, as being fundamentally the same process, differing only in the size of the colloidal aggregates. Both require that there be present an antigenic substance, whether dissolved or in suspension, and an immune serum containing the specific antibody which has been produced by immunizing with this or an equivalent antigen. The general properties of the antigens and the antibodies involved have been considered in previous chapters, and here we shall discuss chiefly the nature of the reactions themselves.

Historically, agglutination came first, having been studied thoroughly first by Gruber and Durham in 1896, although observed previously by others.[3] The discovery of Rudolf Kraus in 1897 that bacterial filtrates

[1] *J. Hyg.*, **14**:261 (1914).
[2] *J. Exp. Med.*, **46**:303 (1927); **48**:183 (1928).
[3] For historical review, *see* Heitz, "Le Phénomène de l'Agglutination," Ernest Claude, Nancy, 1920.

produce precipitates when mixed with the specific antiserum was a logical outcome of the discovery of agglutinins, which was followed in 1899 by the observation that this is a general reaction for other than bacterial proteins, Tschistovitch securing precipitins for eel serum, Bordet for milk and for chicken blood. Agglutination is also exhibited with other than bacterial cells, e.g., red corpuscles, leucocytes, spermatozoa. Although to this day we do not know certainly to what extent agglutination plays a part in protecting the body against infection,[4] or what value the precipitation of foreign proteins has as a means of defense against harmful proteins, the study of these reactions *in vitro* has brought out many facts of both practical and theoretical importance.[5]

Normal Agglutinins and Precipitins

It must be mentioned that normal serum, that is, serum of unimmunized animals, has often the capacity to produce agglutinin and precipitin reactions when not too much diluted. For example, normal human serum diluted not more than ten times will commonly agglutinate typhoid and other bacteria, and give precipitates with undiluted foreign sera and other proteins. Whether these "normal" agglutinins and precipitins are the same as the agents which produce the reactions in much more highly diluted immune sera, is not known. Possibly they represent non-specific antibodies that arise by natural immunization to bacteria and other foreign proteins that enter the body from the intestines, for they are not present early in life.[6] Landsteiner [7] and others [8] believe that they are not specific and are more resistant than the antibodies that result from immunizing.[9]

As yet, however, we have learned chiefly of merely quantitative differences between the agglutinating and precipitating properties of normal and immune serum, but these quantitative differences are very great, for we may, through immunization, increase the activity of a serum until it will produce distinct reactions when diluted 100,000 times or even more. This increased reactivity is obtained on immunizing,

[4] *See* review on "The Functional Rôle of Agglutinins" by Bailey in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928.

[5] *See* discussion of "Bacterial Agglutinins and their Applications" by Fitzgerald and Fraser in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928.

[6] Vorschütz [*Centr. Bakt.*, **90**:105 (1923)] believes that some of the non-specific Widal reactions depend merely on the increase in the globulins of the blood in disease, and are different from the specific reactions.

[7] Landsteiner and Reich, *Z. Hyg.*, **58**:213 (1907).

[8] Heuer, *Z. Hyg. Infektionskrankh.*, **95**:100 (1922).

[9] Boissevain [*Compt. rend. soc. belge biol.*, **2**:165 (1922)] has advanced the hypothesis that specific immune agglutinins are merely nuclei of antigens charged with normal agglutinins.

not merely with pathogenic bacteria, but with any sort of foreign cell or antigenic protein, and, indeed, artificial immunization with bacteria leads to much greater antibody activity in the serum than does infection with the disease itself. For example, in persons with typhoid fever we commonly find the agglutinin reaction given by the serum in dilutions not higher than 1 to 100 or 200; in persons vaccinated against typhoid, usually by three injections, the serum is often active at dilutions of 1 to 500 or even higher, and in immunized laboratory animals, to which we may give as many injections as we like, the serum may agglutinate in dilutions of 1 to 100,000.

Agglutinogens

When agglutinins are produced through infection or after injection of entire cells, it is evident that the agent which incites their formation, i.e., the agglutinogen, must escape from the cell confines. As foreign cells are disintegrated more or less rapidly after injection, by means of processes described elsewhere (see Cytolysis, Opsonins, Phagocytosis), the freeing of the agglutinogen is readily accounted for. By analogy it is safe to assume that agglutinogens are proteins, as the antigens which produce other immunity reactions seem to be. In support of this is the fact that proteins isolated as completely as possible from various cells will, when used as antigens for immunizing, engender antibodies which not only precipitate the antigen but also, under proper conditions, agglutinate specifically the sort of cells from which the antigenic protein was derived.

However, there is evidence that some agglutinogens may, like the toxins, be of smaller molecular dimensions than the ordinary proteins. Since bacteria contained within a collodion sac implanted in an animal give rise to the production of agglutinins in the blood of that animal, it is evident that the agglutinogens are diffusible to some extent, at least through collodion. Agglutinogens in solution are said to pass through certain dialyzing membranes, while agglutinins do not, which suggests that the agglutinogen is of smaller molecular dimensions than the agglutinin, just as toxin molecules seem to be smaller than antitoxin molecules.

Old cultures of bacteria contain free agglutinogens, probably liberated from disintegrated cells, and filtrates of such cultures will neutralize agglutinins, showing both that the agglutinogens are filterable, and that the reaction of agglutinin with the antigen is not dependent upon the presence of cells as such, but upon their soluble constituents.

Formalin, heat, or ultra-violet rays in concentrations just sufficient to kill bacteria do not destroy their agglutinogens,[10] although they may be inactivated by more vigorous treatment which denaturizes proteins. Such killed bacteria are agglutinated readily by immune serum, again showing that this reaction does not depend on the active participation of living cells. Even after heating to 100°, typhoid bacilli bind agglutinin much as if unheated (Lange).[11]

Alterations in the agglutinability of bacteria are marked,[12] e.g., strains of typhoid bacilli freshly cultivated from human infections may be practically inagglutinable even by active serum, but after prolonged cultivation on media they may or may not develop agglutinability. This phenomenon has not yet been fully explained, but it may depend on an active immunity of the bacteria against the agglutinins, for it can be developed in agglutinable strains by cultivating them in serum containing agglutinins.[13] Such non-agglutinable bacteria injected into rabbits produce antisera that will agglutinate ordinary agglutinable strains, but not themselves; hence they do not lack agglutinogens. They give normal complement fixation reactions, and hence do not lack receptors to bind the antibody, and they agglutinate with acids and chemicals much the same as ordinary agglutinable strains.[14] Moreover, identical strains of bacteria grown on media of different composition may show considerable variations in agglutinability.[15] Many strains of bacteria also develop an *autoagglutinative* character, associated with the formation of rough surfaced colonies and often a reduction in virulence.[15a] This agglutination seems to be brought about by salts acting on bacteria that have suffered a reduction in the hydrophilic soluble specific substance that serves to prevent cohesion of the bacteria (White).[15b]

Just what constituent of the bacteria acts as the stimulus to the production of the agglutinin is unknown. It may be assumed, from what we know of antigens in general, that any or all of the protein constituents of the bacteria might serve as the agglutinogen, but according to some of the early investigators (Pick, Joos and Scheller) apparently there are at least two bacterial substances with this property, one of which seems not to be a protein, since it is soluble in alcohol,

[10] Stassano and Lematte, *Compt. rend. Acad. Sci.*, **152**:623 (1911).
[11] *Z. Immunitäts*, **32**:449 (1921).
[12] Bordet, "Studies in Immunity." Bordet and Gay, p. 526. Wiley and Sons, 1909.
[13] Walker, *J. Path. Bact.*, **8**:34 (1902).
[14] McIntosh and McQueen, *J. Hyg.*, **13**:409 (1914).
[15] Dawson, *J. Bact.*, **4**:133 (1919).
[15a] *See* Arkwright, *J. Path. Bact.*, **24**:36 (1921); Hadley, "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, Chapter VII.
[15b] *J. Path. Bact.*, **31**:423 (1928).

gives no biuret reaction and resists temperatures up to 165°. The other gives all protein reactions, and is destroyed by heating to 62°. They considered, therefore, that there are two agglutinogens in the bacterial cell—one, thermostable, the other, thermolabile. More recently it has been found [16] that pneumococci contain a non-antigenic material, apparently a carbohydrate, which gives type-specific reactions with antipneumococcus serum, and also an antigenic protein which gives reactions specific for the species but not for the type of pneumococcus. This probably accounts for the observations on the presence of heat-resistant, non-protein agents in bacteria, which react with immune sera, although not antigenic when injected into animals (see also p. 81).

The question as to whether the agglutinogens occur in the membrane or inside the bacterial cell has been discussed, but Craw found that the insoluble residue of crushed typhoid bacilli, after being washed free of all soluble constituents, was but slightly agglutinated by active serum; therefore, the agglutinogens are possibly soluble intracellular substances.[17] In the case of capsulated bacteria, the capsule seems to be composed of the non-antigenic carbohydrate which reacts specifically with the agglutinins. Stober [18] and Bauer [19] hold that bacterial agglutinogens are lipins, which is disputed by others, but the question of the antigenic capacity of lipins is discussed elsewhere (Chapter II).

Properties of Agglutinins

What has been said previously concerning the properties of the antibodies in general covers the agglutinins, which belong to the group of antibodies described as characterized by producing an altered degree of dispersion of the antigens. The following qualities have been observed specifically in agglutinins: All attempts to separate the agglutinins from proteins have been unsuccessful, and extraction with lipoid solvents of serum which contains agglutinins does not inactivate them.[20] Like most of the other immune substances they are precipitated out of the serum chiefly in the globulin fraction. The isoelectric point of agglutinins lies between 2×10^{-6} and 4×10^{-6} (Szent-Györgi) [21] which agrees with that of serum globulin (3×10^{-6}). However, as all the different methods which precipitate the serum globulins do not

[16] Dochez and Avery, *J. Exp. Med.*, **26**:477 (1917); Heidelberger and Avery, *J. Exp. Med.*, **38**:73 (1923). Review by Heidelberger, *Physiol. Rev.*, **7**:107 (1927).
[17] *See also* Beguet, *Ann. Inst. Pasteur*, **41**:49 (1927).
[18] *Biochem. Z.*, **77**:388 (1916).
[19] *Biochem. Z.*, **83**:120 (1917).
[20] Krumwiede and Noble, *J. Immunol.*, **6**:201 (1921).
[21] *Biochem. Z.*, **113**:36 (1921).

equally precipitate the agglutinins [22] it seems possible that the agglutinins are not the globulins themselves but merely adsorbed substances, the amount adsorbed depending on the pH and salt content of the fluid in which the precipitation is taking place.[23] Typhoid agglutinins dissociated from the bacilli to which they had been united, and further purified, were found by Uchida [24] to contain only a little nitrogen and but little if any demonstrable protein, thus resembling other similarly isolated antibody preparations. Similarly, Flaum [24a] has observed hemagglutinin formation in spinal fluid which contained not enough globulin to give a Pandy test. Kapsenberg and Kispens [25] find that the agglutinin is all contained in the globulins of serum, but equally divided between the several fractions of the globulins, apparently because of adsorption of agglutinins by the globulins. The observations of Bond [26] also suggest that agglutinins may become physically bound to other colloids within the body. Red corpuscles sensitized with specific agglutinating sera behave, when placed in the interspace between oil and water, as if they had received a deposit of protein on the surface (Mudd),[27] this fact strongly supporting the view that the agglutinins are proteins.

Stark [28] found that trypsin does not attack the agglutinins readily, corresponding to the resistance of the serum globulins to this enzyme; alkaline papayotin solution destroys them slowly, while pepsin acts more rapidly. Alkalies are destructive even when quite dilute, while acids are much less harmful. The temperature resistance of agglutinins seems to be variable, plague agglutinin being destroyed at 56°, while most agglutinin sera lose their activity at 60°-65°. Heated sera which have lost their bactericidal activity through destruction of complement (q.v.) still agglutinate bacteria, showing that complement is not essential for the agglutination reaction. The rate of reaction of agglutinins increases with the temperature, as long as this is not high enough to injure the reacting substances.[29] They are readily adsorbed by suspensions, animal charcoal being particularly effective according to Bleyer,[30] who found that the agglutinin could not readily be split off again.

If, when performing an agglutinin reaction, instead of diluting an agglutinating serum with 0.8 per cent salt solution as we usually do

[22] Went, *Z. Immunität.*, **35**:503 (1923).
[23] *See* Schierge for a review on the chemistry of agglutinins, *Z. Immunitäts*, **29**:527 (1920).
[24] *J. Infect. Dis.*, **40**:597 (1927).
[24a] *Acta Path. Microbiol. Scand.*, **5**:16 (1928).
[25] *Z. Immunität.*, **52**:227 (1927).
[26] *Brit. Med. J.*, June 14, **1919**.
[27] *J. Exp. Med.*, **40**:633, 647 (1924); **43**:127 (1926).
[28] *Inaug. Dissert.*, Wurzburg, **1905**.
[29] Madsen *et al.*, *J. Exp. Med.*, **8**:337 (1906).
[30] *Z. Immunität.*, **33**:478 (1922).

in testing its strength, normal serum of the same species is used as the diluting fluid, the optimum agglutinating concentration then observed is much lower than with salt solution dilution, presumably because the serum proteins act as protective colloids and inhibit the agglutination (Heuer).[31]

Specificity with agglutinins is apparently less marked than in other immunological reactions, because since the test is applied necessarily with intact cells we are not dealing with single antigens as we may with other reactions in which isolated proteins can be used. It is to be expected that the complex structure of cells will permit the occurrence of similar or identical proteins, carbohydrates, and lipoids in cells of different species, especially when these are closely related. This may explain the phenomenon of group reactions so commonly seen in testing the agglutination of bacteria, for between closely related species of bacteria the differences are quantitative only. This is shown in the following tables from Karsner and Ecker.[32]

Typhoid immune serum	*B. typhosus*	*B. paratyphosus A*	*B. coli.*
1–4	+	+	+
1–8	+	+	+
1–16	+	+	+
1–32	+	+	—
1–64	+	+	—
1–128	+	—	—
1–512	+	—	—
1–1024	+	—	—
1–2048	+	—	—
1–4096	—	—	—
Salt solution	—	—	—

Agglutination of	Typhoid immune serum	Cholera immune serum
Against *B. typhosus*	1–2000	1–10
Against *B. paratyphosus*	1–100	1–10
Against *B. coli*	1–25	1–10
Against *V. choleræ*	1–10	1–3000

Ehrlich's Concept of the Structure of Agglutinins

The structure of the agglutinins (in the Ehrlich theory) is similar to that of the toxin, i.e., there is a haptophore group by which they combine with the agglutinogen, and a toxophore (agglutinophore) group by which they produce the changes that cause agglutination. The agglutinogen is supposed to be related to the antitoxins in structure,

[31] Heuer, *Z. Hyg.*, **95**:100 (1922).
[32] Principles of Immunology, Lippincott, 1921 pp. 85, 86.

having a single haptophore to unite with the agglutinin. By degeneration of the toxophorous group of the agglutinin, *agglutinoids* may be formed which possess the property of uniting with agglutinogens without producing any agglutination.[33] It was believed by Ehrlich that agglutinins are cell receptors, which have a group with a chemical affinity for the agglutinogen of the bacterial protoplasm, and also another group which brings about the agglutination. They are, therefore, apparently more complex than the simple antitoxic receptors that unite with toxins, and hence were called by him receptors of the second order.

The Principles of the Agglutination Reaction [34]

These have been well discussed by Buchanan [35] and I quote the following passages from his review of this topic: "Before undertaking a consideration of the question, why do bacteria agglutinate, it may be well to ask, why do bacteria *not* agglutinate? An observation of a hanging drop of bacteria will usually show either active motility or active Brownian movement. Cells constantly collide, or at least approach each other closely, then they move apart, apparently repulsing each other, and remain quite uniformly distributed over the field. The factors which may have to do with agglutination are the repulsion of the cells for each other, the vigor of their movements whereby they collide or enter each other's sphere of effective influence, and the attraction which the cells may possess for each other. Agglutination occurs whenever the forces tending to draw these particles together are more powerful than those of repulsion.

"Let us first consider the factors which determine the repulsion of bacterial cells for each other. It has long been known that particles suspended in water usually bear an electric charge. When placed in an electric stream they generally move toward the anode, that is, they bear a negative charge; in a few cases the charge is reversed and they move to the cathode. Hardy showed that the particles of a colloidal suspension of boiled egg-white moved to the anode, but that the direction could be reversed by the presence of suitable electrolytes. This observation was extended to bacterial suspensions by Bechhold who found that bacteria whether heated or unheated wander to the anode. This fact has been abundantly confirmed by other investigators since. Apparently suspensions of bacteria as ordinarily grown are negatively

[33] Olitzky [*Centr. Bakt.*, 106:267 (1928)] says that formalin will destroy the agglutinophore, leaving the haptophore unaltered.

[34] *See also* review by Northrop, Chapter LVIII, "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928.

[35] *J. Bact.*, 4:73 (1919), full bibliography.

charged and the mutual repulsion of the cells may be due to these charges.[36]

"The occurrence of the phenomenon of flocculation or agglutination is in itself evidence that there must exist a force of attraction, under certain circumstances at least. This force is probably surface tension. If the bacterial cells do not repel each other, nor attract each other *per se,* the phenomenon of surface tension[37] explains the fact that they remain together.

"It is evident then, that we have at least a partially adequate explanation of why bacteria under certain conditions remain in suspension, and why under other conditions they may cling together. We may regard the similar electric charge as constituting the repulsing agency, and surface tension as the attracting agency. A study of the agglutination phenomenon then resolves itself into a consideration of the means whereby these two forces may be modified, increased or diminished. Agglutination occurs whenever the similar electric charges are decreased to amounts such that they will no longer overcome the pull of surface tension. Or conversely, surface tension may be increased until it overcomes the dispersion effect of the similar charges."

With these essential facts before us, we may pass to a more detailed consideration of

The Mechanism of Agglutination

This has been a fruitful field of research, in which the application of physical chemistry has been profitable. At first it was believed that the clumping was brought about by loss of motility, until it was found that non-motile bacteria were equally affected. Similarly, the hypothesis of adhesion of the flagella was disposed of.[38] Paltauf considered that the specific precipitin produced by immunization carried the bacilli down in the precipitate formed. In support of this hypothesis is the observation of Scheller[39] that typhoid bacilli in agglutinating serum lose their agglutinability after vigorous shaking, which may be interpreted as the result of disintegration of the agglutinating precipitate.[40]

[36] Webster [*J. Gen. Physiol.*, 7:513 (1925)] has shown that when two types of bacteria are in suspension at a H-ion concentration at which one type is negatively charged and the other is positively charged, agglutination occurs, thus supporting the theory that bacterial repulsion depends on the repulsion of similar charged particles. (H.G.W.)

[37] The terms surface tension, surface energy, and cohesive force have been applied by different investigators to indicate the force that tends to draw the particles together. The rôle of surface tension in bringing bacteria together is not clear. It is safer at present to speak of cohesive force, admitting our present lack of understanding as to what it depends on. (H.G.W.)

[38] For bibliography, *see* Craw, *J. Hyg.*, 5:113 (1905).

[39] *Centr. Bakt.*, 54:150 (1910).

[40] Specific distinct agglutinins for flagella and for bacterial cell bodies have been described. [*See* Arkwright, *J. Path. Bact.*, 30:345 (1927); 31:665 (1928).]

Coplans [41] found the reaction of agglutinin with antigen associated with an increase in conductivity in the solutions, but whether this depends upon the agglutinin reaction itself, or upon associated processes, is questionable. Agglutinated bacteria can be separated from one another by the action of organic and inorganic acids, alkalies, acid salts, and by heating to 70° or 75°, and after once being separated they cannot be reagglutinated by fresh serum.[42]

Neisser and Friedemann [43] found that if the bacterial cells were saturated with lead acetate, washed in water until all soluble lead was removed, and then treated with H_2S, they were promptly agglutinated and precipitated, supporting other observations which indicate that precipitation within the bacterial cells can lead to agglutination. This sort of agglutination is related to the process of formation of coarse flocculi in suspensions, and probably depends upon alterations in surface tension.

Arrhenius [44] has attempted to show that the gas laws are applicable to the partition of agglutinin between the bacteria and the medium, which he compares to the partition of iodin between water and carbon disulfid. This view is not generally accepted. (See Craw [38] and Dreyer and Douglas.[45])

Influence of Electrolytes

Bordet [46] made the important observation that *agglutination does not occur if both the bacterial suspension and the agglutinating serum are dialyzed free from salts before mixing;* but if, to such mixtures, a small amount of NaCl is added, agglutination and precipitation of the bacteria occur at once.[47] This observation brought the phenomenon of bacterial agglutination into close relation with the precipitation of colloids by electrolytes, Bordet comparing it to the precipitation of particles of inorganic matter suspended in the fresh water of rivers that occurs when the fresh water meets the salt water of the ocean. He found that if the agglutinin combined with the bacteria in the absence of the salts, the resulting compound was precipitated by the addition of minute amounts of electrolytes, which alone did not precipitate or agglutinate

[41] *J. Path. Bact.*, **17**:130 (1912).
[42] Eisenberg and Volk, *Z. Hyg.*, **40**:192 (1902).
[43] *Münch. med. Wochschr.*, **51**:465 and 827 (1904).
[44] *Z. physik. Chem.*, **46**:415 (1903).
[45] *Proc. Royal Soc.* (London), **82**:185 (1910).
[46] *Ann. Inst. Pasteur*, **13**:225 (1899).
[47] Porges, however, reported that with very strong immune sera agglutination may occur even when the salt has been removed [*Centr. Bakt.*, **40**:133 (1905)], but Northrop and De Kruif [*J. Gen. Physiol.*, **4**:655 (1922)] found that under these conditions agglutination is not complete, the effect being about the same as with dialyzed normal serum.

the bacteria or the serum. This is a general principle applying not only to the agglutination of bacteria, but also of other cells.[48] It indicates that the agglutinins cause a change in the cells which bring them under the same physical laws as the inorganic colloidal suspensions, which are characterized by being precipitated by the addition of traces of electrolytes. This corresponds to the situation observed when to a solution of a colloid is added another colloid of opposite charge, but in too small an amount to bring about flocculation, for the further addition of even a small amount of salt may then produce flocculation.

Therefore, in agglutination there are two distinguishable phases, the fixation of the antibody to the cells and the subsequent coming together of the cells. This fixation, says Nicolle,[49] follows the rule foreseen by Bordet and expressed by Eisenberg and Volk in these terms: For the same mass of cells the absolute quantity fixed is directly proportional to the concentration of the antibody; the relative quantity, inversely. This rule, valid for the action of all antibodies, is the contrary of Dalton's chemical law, but it corresponds to van Bemelen's principle which governs the adsorption of colloids.

Resemblance to Colloidal Reactions

In all physico-chemical respects the behavior of bacteria and agglutinin resembles the behavior of colloidal mixtures which form an electrically amphoteric colloidal suspension, so that the ions of electrolytes or the electric currents cause precipitation by discharging them (Neisser and Friedemann).[43 50] This is well illustrated by the tables on page 158. The first shows how the sensitization of a gum mastic solution by a minute quantity of gelatin renders it readily precipitable by very small amounts of NaCl, just as bacteria sensitized by agglutinin are flocculated by the small quantity of salt present in the suspension. The second table illustrates the similarity of the zone phenomenon in simple colloidal reactions with that seen in agglutination and precipitation tests when an excess of antigen fails to produce reactions which are given by weaker concentrations.

Neisser and Friedemann,[43] Bechhold,[51] and Friedberger[52] investigated the precipitation of inorganic suspensions and of colloids by several salts, examined the limiting concentration of salt solution in

[48] Eisner and Friedemann, *Z. Immunitäts.*, **21**:520 (1914).
[49] *J. State Med.*, **28**:293 (1920).
[50] *See also* Girard-Mangin and Henri, *Compt. rend. soc. biol.*, **56**:866 (1904); and Zangger, *Cent. Bakt.* (ref.), **36**:225 (1905); Günter, *Arch. Hyg.*, **92**:211 (1923).
[51] *Z. physik. Chem.*, **48**:385 (1904).
[52] *Centr. Bakt.*, **30**:336 (1901).

NaCl 10% solution	1 cc. mastic (1-10 original emulsion) diluted to 3 cc.	1 cc. mastic + 0.0001 cc. of a 2% gelatin solution, the whole diluted to 3 cc.
1.0	+++	+++
0.5	0	+++
0.25	0	+++
0.125	0	+++
0.05	0	0
0.025	0	0

Colloidal iron hydroxid	Precipitation of mastic emulsion, 1 cc.
1.0	0
0.5	0
0.25	0
0.1	++
0.05	+++
0.025	+++
0.01	+++
0.005	+++
0.0025	++
0.001	0

which the action of precipitation took place in 24 hours, and proved that the really important factor in the action of salts on bacteria is the cation and not the anion, presumably because bacteria are electro-negative. In addition to the agglutinin and bacteria, therefore, the presence of some cation is necessary for the mechanism of agglutination. Shionoya [53] found that the more the valency of the cation increases, the less becomes the lowest concentration at which agglutination takes place.

Agglutination obeys the same laws as other similar physical phenomena; the rate of agglutination depends upon the concentration of the suspension and of the electrolytes, and varies with the valence of the cations. Although bacteria in an electric stream move toward the anode, Bechhold [51] reported that after being acted on by agglutinin they are agglutinated by the current between the poles; which he thought indicated the importance of the electrical charges of the bacterial surfaces in their agglutination reactions. However, Buxton and Teague [54] and subsequent observers found that agglutinin-bacteria do move towards the anode, but more slowly than normal bacteria.

Before the agglutinin combines with the cells they behave like the colloidal solutions of organic colloids, being precipitated only by the salts of heavy metals, alcohol, formalin, etc., or by high concentrations of neutral salts. Field and Teague [55] found that agglutinins carry

[53] *Lancet*, 1922, Oct. 28, p. 905.
[54] *Kolloid Z.*, 1908, II, Suppl. 2.
[55] *J. Exp. Med.*, 9:86 (1907).

positive charges while bacteria are negative, and that by the electric current agglutinins can be separated from bacteria with which they have combined; this shows that the agglutinin is not destroyed in the reaction.[56] But as far back as 1907 Teague and Buxton [57] considered that neutralization of the electric charge of the bacteria is not the only important factor in agglutination, a view in harmony with present-day opinion.

Effect of H-ion Concentration

Michaelis and Davidsohn [58] found that specific agglutination and precipitation reactions can occur within a considerable range of H-ion concentration, the optimum reaction not corresponding with the isoelectric point of either the antigen or the serum globulin which contains the antibody; only with very dilute antigen or antibody solutions does the pH have much influence, and then the results are best at about the neutral point. These facts have been interpreted as indicating that the electric charge of the reacting agents cannot be an important factor in the production of these specific reactions. DeKruif and Northrop [59] have found that the amount of immune body combining with bacteria is constant within a range of pH 9 to pH 3.7. They consider that their results are contradictory to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and by the bacteria, but fit better the assumption that the immune body forms a film on the surface of the bacteria. This film is responsible for the change observed in the bacterial charge after the bacteria have been acted upon by immune bodies. They also found that the amount of immune serum required to agglutinate bacteria depends on the pH of the mixture, the nearer this is to the acid agglutination zone of the bacteria the less serum being required to agglutinate them.

Surface Adsorption of Agglutinins

Dean [60] interprets agglutination as follows: The process of agglutination is probably brought about by an interaction between the antiserum and antigen which has passed out from the bacillus into the medium which immediately surrounds it. The result of this interaction is the aggregation of the particles of the globulin of the antiserum on

[56] Because of this negative charge on bacteria they are agglutinated by basic dyes, but not by acid dyes, there being no specific action by different basic dyes [Brossa, *Z. Immunitäts.*, 37:221 (1923)].
[57] *Z. physik. Chem.*, **57**:76 (1907).
[58] *Biochem. Z.*, **47**:59 (1912).
[59] *J. Gen. Physiol.*, **5**:127 (1922).
[60] *Lancet*, Jan. 13, 1917, p. 45.

the surface of the bacillus. In other words, the proteins of the antiserum are adsorbed by the bacillus.[61] For the production of agglutination the presence of precipitable protein is essential. If to a mixture of bacillary emulsion and diluted antiserum there be added a third ingredient, normal serum, the agglutination of the bacteria is rendered more complete. This reinforcement of the proteins of the antiserum by those of a normal serum has been called *conglutination*.

Coulter's observations [62] with hemagglutinins support Dean's views, for he found that the optimum pH for agglutination of red corpuscles in sucrose solution is 4.75, which is their isoelectric point, but when they are sensitized the optimum pH is 5.3, which is probably connected with the optimum for flocculation of the immune body, for this is the isoelectric point for the euglobulin of the immune serum. Apparently in sensitization of corpuscles by antibodies there is a condensation of euglobulin on the surface of the cells. This is endorsed by the work of Shibley,[63] which indicates that in the sensitization of bacteria they become coated with the globulin of the antibody. This gives them the character of particles of denatured protein which follow the laws governing denatured proteins and flocculate whenever their charge is reduced by electrolytes to a critical level lying somewhere between 12 and 14 millivolts.

Alterations Produced by Agglutinins

According to Bechhold,[64] normal bacteria behave like inorganic suspensions that have each particle protected by an albumin-like membrane, which prevents them from being thrown out of suspension by solutions of alkali salts, etc. After being acted on by agglutinin they are so altered that they behave like the unprotected inorganic suspensions, and are precipitated by salts and other electrolytes. This suggests the possibility that the agglutinin makes the bacteria permeable for these electrolytes. Buxton and Shaffer [65] also found that bacteria which have been acted upon by agglutinin behave as if their proteins had been so changed that they are more capable of adsorbing or combining with salts than when in their normal condition. Strong salt solutions inhibit agglutination, apparently by preventing the binding of the agglutinin.[66] Tulloch [67] observed that in the presence of salts of mono-

[61] *See also* Mudd, *J. Exp. Med.*, **43**:127 (1926).
[62] *J. Gen. Physiol.*, **3**:309, 515 (1922); **4**:403 (1922).
[63] *J. Exp. Med.*, **44**:667 (1926).
[64] *Z. physik. Chem.*, **48**:385 (1904).
[65] *Z. physik. Chem.*, **57**:47 (1907).
[66] Landsteiner and St. Welecki, *Z. Immunitäts.*, **8**:397 (1910).
[67] *Biochem. J.*, **8**:293 (1914).

and di-valent cations, unsensitized bacteria do not readily precipitate or agglutinate, but bacteria sensitized with agglutinins, as Bordet showed, agglutinate with small quantities of such salts. In this respect unsensitized bacteria behave like "non-rigid colloids," such as fresh egg white, while sensitized bacteria resemble "rigid colloids," such as denatured egg white. Hence he advances the hypothesis that the process of sensitization is akin to that of denaturation of proteins, the specificity perhaps depending on different degrees of denaturation.[68]

Mansfield [69] would bring agglutination into line with other serological reactions as a protein digestion process, by his hypothesis that bacteria are held in suspension by protective colloids which are digested by an enzyme, which is the agglutinin. He finds in favor of this hypothesis that the temperature and reaction curves correspond to enzyme actions, that agglutinating serum contains an enzyme digesting protein extracted from bacteria, and that during agglutination the agglutinogen is destroyed. This hypothesis does not seem to have been reinvestigated, but it is supported by Szent-Györgi.[70] It assumes that bacteria may be looked upon as suspension colloids which are protected from flocculation by having each particle surrounded by a protective colloid which is the agglutinogen. The agglutinin is believed to act by digesting or otherwise altering this protective colloid so that it no longer protects, whereupon the bacteria lose their electrical charges through adsorption of ions from the neutral salts present in the solution. When the electrical charge is lost the mutually repellent action of the like electrical charges of the bacteria is lost, hence they come together until in large enough quantity to succumb to gravitation and settle out of suspension. The protective colloid also interferes with chemical action in general, and when it is destroyed by the agglutinin the permeability of the bacteria for various ions, including bactericidal agencies, is increased, hence agglutinins may play a part in defense over and above that of agglutinating the bacteria.

Ionization of Antigens

As E. F. Hirsch [71] points out, Bordet's observation that the presence of a salt is necessary for agglutination may be taken to mean

[68] The chief objection to Bordet's views of the significance of electrolytes in agglutination lies in the observation of Friedberger that such a crystalloid non-electrolyte as dextrose agglutinates sensitized bacteria; but this occurs only in higher concentrations than are adequate with electrolytes; furthermore, milk sugar and urea have no such effect, which throws doubt on the significance of the glucose result.

[69] *Z. Immunitäts.*, **27**:197 (1918).

[70] *Z. Immunitäts.*, **30**:144 (1920).

[71] *J. Infect. Dis.*, **30**:651 (1922).

that in distilled water the bacterial protein is not ionized to an appreciable extent. In order to react with immune serum and agglutinate, ionization is necessary and occurs when the bacterial protein is combined with a base to form a salt. Hirsch's study of agglutination indicated that bacteria suspended in normal salt solution behave chemically and electrically like the anion of the salt of a strong base and a weak acid. When bacteria are agglutinated by immune serum, the medium in which this reaction occurs increases in alkalinity. This change in reaction is believed to result from differences in the dissociation constants of the reacting substances and their products. Hirsch therefore suggests that the agglutination of bacteria is associated with the neutralization of their electrical charges by the immune substance, the behavior of which indicates that it carries a positive electrical charge, that it possesses basic properties, and that it ionizes according to a definite formula. "The neutralization of the negative charge of the bacteria by the immune body liberates the cation of the bacteria (Na) and the anion of the immune body (OH). The dissociation constant of the sodium hydroxide resulting from this interaction is probably so much greater than the dissociation constant of the immune substance that more hydroxyl ions are contained in the liquid after agglutination than before, and the medium becomes more alkaline."

Acid Agglutination

Physico-chemical researches have as yet failed to explain the specific character of the agglutinins for specific bacteria, but Michaelis [72] developed an interesting analogy in the specific agglutination of bacteria by acids. This is based on the fact that the optimum concentration of H-ions which precipitates proteins from solution is characteristic and constant for each protein, and the same is to some extent true for the agglutination of bacteria by acids. Some of the early investigators reported that the agglutination by acids may be even more sharply specific than the agglutination by immune sera; e.g., typhoid and paratyphoid bacilli were said to be readily distinguished, because the former are agglutinated by a concentration of H-ions from 4 to 8×10^{-5}, while paratyphoids require 16 to 32×10^{-5}, and colon bacilli are not readily agglutinated by acids. The acid agglutination, however, does not always affect all strains in the same way, some strains which are not readily agglutinable by antisera also resisting acid agglutination.[73]

[72] *Folia Serologica*, 7:1010 (1911); also Beniasch, *Z. Immunitäts.*, 12:268 (1912).
[73] *See* Kemper, *J. Infect. Dis.*, 18:209 (1916).

Bacteria which have been sensitized by serum are more sensitive to acid agglutination than are normal bacteria.[74] According to Arkwright,[75] typhoid bacilli contain two extractable proteins that are agglutinated by acids, one at 3.6×10^{-5} and the other at 1.1×10^{-3}; the former seems to be related to, if not identical with, the substance that is precipitated by immune serum. Apparently the agglutinable substances of bacterial flagella and of bacterial cells have distinct differences in the H-ion concentration at which they are agglutinated.[75a] Acid agglutination of bacteria apparently belongs to the same class of reactions as the coagulation by H-ions of amphoteric colloids of preponderatingly acid character and does not seem to have been found by later investigators to be as specific as Michaelis and others maintained.[76]

The Influence of Surface Tension and Electrical Potential on Agglutination

Modern investigation on the behavior of colloids serves further to emphasize the importance of the pH and the salt content of the solutions in which immunological reactions are taking place, neglect of these undoubtedly being responsible for many errors in the past. The possibilities of such factors are especially well brought out by the important studies of Northrop and DeKruif,[77] from which I have abstracted freely in the following paragraphs. These authors have investigated the *cohesive forces* which may be a factor in agglutination, as well as the electrical charge, for agglutination of bacteria by immune serum may occur without any measurable change in the electrical potential.

Suspension of bacteria or other particles is maintained by the repellent action of the like electrical charges[78] which they carry, and which opposes the force of attraction exerted, supposedly, by their surface tension, or, as these authors prefer to say, the cohesive force. Whenever the force of attraction becomes greater than the repulsive force the particles agglutinate, and if the increase of the size of the particles is sufficient they precipitate. Although in the case of simple protein suspensions it is found that agglutination occurs at the point at

[74] Krumwiede and Pratt, *Z. Immunitäts.*, 16:517 (1913).
[75] *Z. Immunitäts.*, 22:396 (1914); *J. Hyg.*, 14:261 (1914).
[75a] Arkwright, *J. Path. Bact.*, 31:665 (1928).
[76] A full review with bibliography is given by Gouwens, *J. Infect. Dis.*, 33:113 (1923).
[77] *J. Gen. Physiol.*, 4:639, 655 (1922).
[78] The term electrical charge as used here and subsequently applies only to the difference in potential between the surface of the bacterium and the suspending fluid as measured by electrophoresis (the electrokinetic potential) and not the Nernst electromotive or thermodynamic potential [*see* Freundlich and Gyemant, *Z. physik. Chem.*, 100:182 (1922)].

which they carry no electric charge, the isoelectric point, in the case of bacteria the results of investigations of their behavior have been much less satisfactory. It was found by Bechhold,[64] Arkwright,[79] Teague and Buxton [80] and others that bacteria were always negatively charged whether or not they were agglutinated. These authors concluded, therefore, that the potential carried by the organisms could not account for the agglutination phenomena.

Northrop and DeKruif undertook to measure both the force which tends to cause the particles to adhere as well as that which keeps them apart, since if both forces are affected by the conditions of the experiment but only one is measured, it will be impossible to interpret the results. The potential may be conveniently measured by the rate of migration in an electric field. The attractive forces, however, have usually been assumed to remain constant and no attempt has been made to measure them. It was found, in the course of their experiments, that a comparative measure of the attractive forces between the organisms could be obtained by measuring the force required to tear apart two glass plates covered with a film of the bacteria and immersed in the solution which was under investigation. As a result of these measurements in conjunction with the measurements of the potential difference, it has been found that whenever the potential difference between the surface of the bacteria which they studied and the solution in which they are immersed is less than about 15 millivolts (either positive or negative) the bacteria agglutinate, provided the cohesive force is not affected. If the cohesive force is decreasd, this critical potential is decreased, and if the cohesive force is made very small, no agglutination occurs even though the potential be reduced to zero. It was further found that all electrolytes tested in concentrations less than 0.01 to 0.1 N affect the potential, but have little effect upon the cohesive force. The cohesive force is influenced by electrolytes only when in a concentration greater than 0.1 N. In the case of bacteria sensitized with immune serum, the cohesive force remains constant and the agglutination can be predicted solely from the measurement of the potential.

Experiments were carried out with the bacillus of rabbit septicemia and *B. typhosus*. When salts or acids were added to a suspension of washed bacilli, it was found that in all experiments there is complete agglutination as soon as the potential is reduced below a value of

[79] Arkwright, *J. Hyg.*, 14:261 (1914).
[80] *Z. physik. Chem.*, 57:76 (1907).

about 15 millivolts (either positive or negative), provided the salt concentration is below 0.001 N. Below this salt concentration, therefore, the agglutination is seen to depend solely on the potential, and any substance which reduces the potential below about 15 millivolts will cause agglutination. There is another range of salt concentration above 0.10 N in which no agglutination occurs, although there is no measurable potential. Between these two ranges of salt concentration there is a zone in which agglutination occurs at various potential levels. This is evidently the result that we would expect if the salt acted in low concentration primarily on the potential, and in high concentration on the cohesive force. There would be an intermediate zone in which the agglutination could not be predicted from either measurement alone. The effect on the cohesion is not connected with the valency nor with the electrical effects of the ions. $LaCl_3$ is far more effective than NaCl in reducing the potential, but less effective in reducing the cohesive force. The agglutination depends on both factors. It is possible, therefore, for all monovalent ions to affect the potential in the same way but to differ in their agglutinating power. In order to predict the efficiency of a salt, it is therefore necessary to know the effect on both the potential and the cohesion.

The effect of the salts on the potential is due to the oppositely charged ion and increases in general with the valence of the ion. The effect is not purely due to the valence since the hydrogen ion is far more active than the other monovalent ions. The result also depends on the nature of the suspension, since the charge on the bacillus of rabbit septicemia may be reversed by sulfate or NaCl, while with *Bacillus typhosus* suspension the charge is reduced but does not change in sign.

The Donnan Equilibrium [81]

As to the origin of the potential, Loeb has shown,[82] in the case of a protein solution separated from a solution of electrolyte by a collodion membrane, that the charge on the protein solution can be almost quantitatively accounted for on the basis of Donnan's theory of membrane potentials. Donnan's theory postulates that when a membrane separates two solutions, the membrane being permeable to all the ionic constituents of one solution and to a part of the ionic and molecular constituents of the other solution, a definite potential difference should

[81] Review by Hitchcock, *Physiol. Rev.*, 4:505 (1924).
[82] Loeb, J., *J. Gen. Physiol.*, 3:667 (1920-21); 4:351 (1921-22); "Proteins and the Theory of Colloidal Behavior," New York and London, McGraw-Hill Book Co., 1922, p. 120.

appear between the solutions when at equilibrium, depending on the concentrations and the charge of the various constituents. Using gelatin and other proteins as the non-diffusible constituent and various electrolytes as the diffusible constituents, Loeb showed that the quantitative results obtained in terms of potential difference are approximately what would be predicted on the basis of the Donnan equilibrium. Northrop suggests that the potential at the surface of the bacterial cell may also be due to a Donnan equilibrium, and the observed results are in agreement with this assumption. However, all particles in suspension are charged, and in many sorts of suspensions the potential cannot be ascribed to a Donnan equilibrium.

Northrop and DeKruif suggest that electrolytes affect the potential of a particle in two ways. (1) By combining chemically with the particle (for example, hydrogen ions). The ion then becomes part of the molecule of which the particle (membrane) is composed. As a result the concentration of this ion differs on the opposite sides of the membrane and gives rise to a potential. This potential may be calculated by Nernst's formula from the concentration of the common ion on both sides of the membrane. (2) By producing ions which affect the distribution of the common ion without further chemical combination with the membrane. This mechanism will suffice to account for all the observations made in the course of Northrop and DeKruif's work, if it be supposed that other ions than the hydrogen ion may act by chemical combination.

To summarize Northrop and DeKruif's experiments, electrolytes in low concentration (0.01 N) affect primarily the potential, and noticeably decrease the cohesive force only in higher concentration (above 0.1 N). As long as the cohesive force is not affected, agglutination of the bacteria studied by them occurs whenever the potential is reduced below about fifteen millivolts. When the cohesive force is decreased the critical potential is also decreased, and hence in concentrated salt solution no agglutination occurs even though there is no measurable potential.[83]

The behavior of bacteria suspended in a salt solution corresponds with the postulates of the Donnan equilibrium for the behavior of colloidal suspensions generally, according to the observations of

[83] Mathematical considerations led Green and Halvorson [*J. Infectious Diseases*, **35**:5 (1914)] to the conclusion that the surface energy or surface tension not the difference in potential between bacterial cells and the surrounding liquid, is the fundamental factor in the stability of suspensions that may either hold the cells apart or clump them together. They state that the charge on such cells must be of the nature of a Helmholtz double layer, which will affect the stability of the suspension only so far as it affects the surface energies, and they interpret the findings of Northrop and DeKruif on this basis.

Winslow, Falk and Caulfield.[84] That the phenomena involved when bacterial cells are suspended in water and salt solutions of the types studied are essentially similar to those observed by Loeb for gelatin and other types of particles, and are in general accord with the Donnan equilibrium, would seem to be indicated by the general parallelism between the results of these authors and those obtained by Loeb. They also confirmed the observations of Loeb and of Northrop and DeKruif that the presence of salts depresses velocity of migration, just as it would depress electrokinetic potential according to the Donnan theory and this depression is particularly effective on the alkaline side of the pH range; also the findings of Northrop and DeKruif in regard to the relatively high depressing effect of $CaCl_2$ as compared with NaCl which is another corollary of the Donnan theory. All of the phenomena observed by them appear to be in accord with the assumption that marked differences in dielectric constants did not appear under the conditions studied and if this assumption be granted the results are in accord with the fundamental postulates of the Donnan equilibrium as applied to the explanation of the origin of potential difference between a bacterial cell and its enveloping menstruum.

Surface Effects of Agglutinins

Northrop and DeKruif also investigated the effect of proteins, normal serum and immune serum on bacteria in respect to the cohesive force and the electrical potential, for it is well known that the addition of a small amount of certain substances, especially proteins, markedly affects the behavior of suspensions. They found that when dialyzed immune serum is added to bacteria in distilled water their potential is gradually decreased, but not sufficiently to cause agglutination. If now salt is added, as in the Bordet experiment, until the potential is decreased to 15 millivolts (either positive or negative), agglutination occurs, indicating that the serum in some way prevents the salt from decreasing the cohesive force and so agglutination takes place whenever the electrical potential reaches the critical point of 15 millivolts. In support of this interpretation is the fact that if to bacteria in suspension in the presence of salt sufficient to reduce the potential to 15 millivolts, immune serum is added, agglutination occurs, for the serum increases the cohesive force until it overcomes the repulsion forces between the bacteria at this potential. The behavior is much as if the agglutinin forms a film on the surface of the organism, which prevents

[84] *J. Gen. Physiol.*, 6:177 (1923).

the inhibiting action of strong salt solutions on the cohesive force of the bacilli. It is evident from the foregoing that the agglutination may be considered as caused by the salt, as Bordet stated. The serum, however, does not sensitize the bacterium but protects it from the salt, so that the latter does not reduce the cohesive force.

Shibley [85] has found that immune agglutinating sera possess a specific charge-reducing effect which is quantitatively related to the agglutinin titer of the serum, disappearing when the agglutin is removed through adsorption by the specific antigen. Addition of immune serum in excess brought the potential of all bacteria studied to the same point, which might be higher, lower, or the same as the original suspension. This potential agreed closely with that of denatured serum globulins, indicating strongly that sensitization of bacteria depends on the formation of a film of serum globulin on the bacterial surface. However, specific agglutination may occur independently of charge reduction. Highly protective antisera which are not agglutinative do not exhibit the specific charge-reducing effect. The findings of Northrop and DeKruif, that unsensitized and sensitized bacteria agglutinate only when the charge on the bacteria has been reduced to a critical potential zone lying between + 15 and — 15 millivolts, were confirmed in the case of all electrolytes tested except Na_2HPO_4. When Na_2HPO_4 or phosphate buffer solutions were used as electrolytes, specific bacterial agglutination occurred at negative charges well above — 15 millivolts, and with serum in high dilution, specific agglutination took place without any observable reduction of charge.

The Problem of Specificity.—These experiments seem to represent a closer approach than we have formerly had to an exact study of the physical forces at play in agglutination, although as yet they offer no explanation as to why immune serum has a greater effect on cohesive force than normal serum, nor do they suggest a clue to the specificity of the agglutination reaction. Northrup [34] dismisses the dilemma of specificity in these words:

"The agglutination of bacteria by their homologous antibody (agglutinin) differs from that of agglutination by ordinary proteins or the agglutination of collodion particles by means of egg albumin in that the antibody will form a film only on the bacteria used to produce it. The specific part of the reaction, therefore, lies in the formation of the film. This specificity is of the greatest theoretical and practical importance but does not differ, in the writer's opinion, from the specificity

[85] *J. Exp. Med.*, 40:453 (1924).

of any chemical reaction. Quantitatively every chemical reaction is specific, and the specificity of immune and enzyme reactions differs only in degree from that of any other reaction. The problem of specificity is one of reactions in general. It is possible with our present knowledge to predict whether or not a given reaction will take place from considerations of the free energy changes involved, but there is no means of predicting the rate at which it will occur. Practically, therefore, it is not possible to predict with certainty the occurrence of even the simplest reaction, so that it is not surprising that such complicated reactions as those involved in biological systems are still unaccounted for."

Hemagglutination [86]

This is merely one example of agglutination of cells under the influence of serum components, and apparently does not differ in principle from the agglutination of bacteria or other cells. It possesses much practical importance, however, because of the fact that a serum may contain hemagglutinins for human corpuscles, and hence if introduced intravenously cause fatal accidents through occlusion of the vessels by clumped red corpuscles. Not only foreign sera but even normal human blood may contain such hemagglutinins for the blood of another individual, and hence the blood of both donor and recipient must be tested as to agglutination before transfusion of blood can be safely performed. The serum of an animal immunized against red corpuscles of another animal usually exhibits such a strong hemolytic effect that its agglutinative activity is not evident, unless the serum has been inactivated by heating at 55°, which destroys the lytic agent (complement) and leaves the agglutinin still active.[87]

Certain points of chemical interest concerning hemagglutination may be worth mentioning. One is that corpuscles which have been hardened by the action of formaldehyde or $HgCl_2$ are still agglutinable, a fact which suggests that perhaps the proteins, with which these chemicals combine, may not be responsible for the agglutination, and there is reason to believe that the lipoid elements of the stroma may play an important part.

Silicic acid and certain other *colloids* may act as agglutinins, their effect bearing a relation to the effects of electrical charges upon agglu-

[86] For full review *see* Sachs and Klopstock, "Methoden der Hämolyseforschung," Berlin, Urban and Schwarzenberg, 1928.

[87] Coulter [*J. Gen. Physiol.*, 10:545 (1927)] states that hemolytic and hemagglutinative properties of immune serum must depend on different properties of the protein, since extraction of the serum globulin with alcohol removes most of the hemolytic activity while the hemagglutinative effect is increased.

tination of cells or of colloids (q.v.).[88] Corpuscles that have been sensitized by hemolytic amboceptors were found by Eisner and Friedemann [89] to be much more readily agglutinated by salts of heavy metals, especially copper and zinc, presumably because of quantitative alterations in the electrical charge of the corpuscles induced by the antibody. Later observers,[90] however, have found these effects much less regular than these authors described them to be, there being different effects with the same metals with the corpuscles of different species.[91]

Northrop and Freund [92] have studied the physical chemistry of hemagglutination, and state that "the agglutination of a number of suspensions has been found to be closely related to the potential difference between the particle and the surrounding liquid as measured by the migration in an electric field. If the potential is higher than a certain value—the critical potential—the particles remain separate; if it is lower than this value, they adhere or agglutinate. It follows that agglutination may be caused in two ways, (1) by decreasing the actual potential, or (2) by increasing the critical potential. In general, electrolytes do not affect the value of the critical potential, so that in most cases agglutination occurs whenever sufficient electrolyte has been added to depress the potential below a certain definite value."

They had previously found that bacteria sensitized by an immune serum agglutinate because the critical potential has been increased thereby, and a similar condition was found to be true with hemagglutination. That is, unsensitized red cells did not become agglutinated until the potential had been depressed below about 4 millivolts, whereas cells sensitized by agglutinating serum agglutinated when the potential was reduced to about 12 millivolts.[93]

Coulter [94] found that the agglutination of red cells in an isotonic saccharose solution is at an optimum when the pH is 4.75, but when these cells have been acted on by immune serum the optimum is at 5.3, and with normal agglutinins it is at 5.5. These latter figures correspond closely to the isoelectric point of euglobulin, and as the antibodies are found associated with globulins it seems probable that the agglutination of the cells is associated with the precipitation of the antibody, furnishing another point of evidence of the essential identity of the precipitins

[88] Landsteiner and Jagic, *Münch. med. Wochschr.*, 51:1185 (1904).
[89] *Z. Immunitäts.*, 21:520 (1914).
[90] W. Schürmann and Fr. Baumgärtel, *Z. Immunitäts.*, 31:151 (1921).
[91] Basic dyes, but not acid dyes, agglutinate erythrocytes [Gutmann, *Centr. Bakt.*, 106:68 (1928)].
[92] *J. Gen. Physiol.*, 6:603 (1924).
[93] Schroeder [*Arch. ges. Physiol.*, 215:32 (1926)] found that in *isoagglutination* of red cells there likewise occurs a decrease in the charge on the cells.
[94] *J. Gen. Physiol.*, 4:403 (1922).

and agglutinins. Presumably the antibodies collect on the surface of the antigenic cells, for it would be difficult to explain how the small amount of serum protein relative to the mass of cells could give its own point of optimum flocculation to a mixture of cells and serum in any other way than as a surface deposition. The behavior of sensitized red corpuscles in the boundary surface between oil and water indicates, according to Mudd,[95] that the agglutinins are proteins which combine predominantly with the protein of the surface of the corpuscles. Presumably the same is true of bacterial agglutinins, and it is of interest that Freund [96] has found that even the fat-rich tubercle bacilli behave in agglutination as if they had a protein surface.

A phenomenon based, as far as one may judge, upon the same mechanism of the combination of a protein with a surface, has been observed by Loeb,[97] who found that collodion membranes always acquire the characteristics of the protein with which they have been brought into contact, and that if such a membrane be treated with a solution of gelatin or oxyhemoglobin, for example, after the surplus protein has been washed away, the isoelectric point of the membrane is now that of the protein with which it has been treated.[98]

It seems probable that the unclotted fibrinogen remaining in serum may be of importance in determining the rate and amount of hemagglutination, especially by normal sera,[99] for if such an active serum exhibiting this so-called *conglutinin* effect is treated with blood platelets to remove this residual fibrinogen, the conglutinin effect is also removed.[100]

Certain *vegetable poisons* produce agglutination of red corpuscles, especially ricin, abrin, and crotin, and the fact that ricin has little or no hemolytic action shows the independence of the processes. The hemagglutinative activity of ricin can be extracted quantitatively from a ricin solution by red corpuscles.[101] Antisera for these vegetable poisons are also antiagglutinative, acting, as Ehrlich showed, on the poisons and not on the corpuscles. The seeds of many non-poisonous leguminous plants, and also of certain *Solanaceæ,* yield extracts that are strongly agglutinative for red corpuscles; in *Phaseolus multiflorus* the active substance is found in the proteose of the seed, and seems to be a part

[95] Mudd and Mudd, *J. Exp. Med.,* 43:127 (1926).
[96] *Am. Rev. Tuberc.,* 12:124 (1925).
[97] *J. Gen. Physiol.,* 2:577 (1919-20).
[98] *J. Exp. Med.,* 46:303 (1927); 48:183 (1928).
[99] Bechhold, *Umschau,* 26:177 (1922).
[100] Maltaner and Johnston, *J. Immunol.,* 6:349 (1921).
[101] Hirsch, *Fermentforschung,* 2:269 and 290 (1919).

of the stored food (Schneider).[102] It is not present in other parts of the plant. The hemagglutinin present in soy bean is readily adsorbed from solutions by kaolin, etc., and may be set free from the adsorbent by weak alkalies, thus yielding a relatively pure solution.[103]

Snake venoms contain hemagglutinins, destroyed by heating to 75°; their agglutinating power being in inverse ratio to their hemolytic power. Corpuscles agglutinated by venoms may be again separated by potassium permanganate solutions.[104]

Conglutination.—Under this term Bordet and Gay described the observation that in ox serum there is a substance which combines with corpuscles (or bacteria) that have been acted upon by agglutinating sera, and augments the agglutination. Dean[105] finds that, in general, agglutination requires two agents, one being the specific antibody, and the other a precipitable substance, probably a globulin. When cells have combined with the antibody the precipitable substance is aggregated on their surfaces, and, presumably, determines the agglutination. More recent studies by Maltaner and Johnston,[100] indicate that the conglutination effect of beef serum is due to the presence of fibrinogen, which is only partly removed in the clotting of blood, especially in the case of beef blood. *Co-agglutination* described by Bordet and Gengou as the agglutination by an antigen and the homologous antibody, of the corpuscles of another animal, is probably closely related to these phenomena (Dean).

THE PRECIPITIN REACTION

As previously stated, this seems to be essentially the same as the agglutination reaction except in the fact that the reaction is demonstrated by the precipitation of a colloidal antigen from solution instead of by the clumping of cells. Therefore what has been said concerning antigens in general and the agglutination reaction in particular, applies to the precipitin reaction, except for certain details not observable in the agglutination of cells by the action of specific antibodies.[106] The precipitin reaction occurs under exactly the same conditions as the agglutinin reaction, electrolytes being necessary to cause the flocculation

[102] *J. Biol. Chem.*, 11:47 (1912).
[103] Fujiwara, K., *Biochem. Z.*, 140:113 (1923).
[104] Flexner, *Univ. Penn. Med. Bull.*, 15:324 and 361 (1902).
[105] Literature given by Dean, *Proc. Royal Soc. (London)* (B) 84:416 (1911); Hall, *Univ. Calif. Pub. Pathol.*, 2:111 (1913); Leschly, *Z. Immunitäts.*, 25:219 (1916).
[106] Felton and Bailey [*J. Immunol.*, 11:197 (1926)] call attention to the fact that a single purified protein fraction of the pneumococcus serves as an antigen to produce precipitins and agglutinins, as well as all the other known antibodies.

of the antigen and antibody molecules after their reaction with each other.

For the biological chemist the precipitin reaction is often of great value in determining the nature of material present in minute quantities, for although the biuret reaction and most other protein tests will not detect a protein in much less than one part in a thousand, the precipitin test will show not only the presence but also the character of a protein in a dilution ten to one hundred times greater. But even this is much less delicate than the anaphylaxis and complement fixation tests.

At present it is not established that precipitins can be secured against lipoids or other non-protein substances. Possibly precipitins can be produced for colloidal substances with molecules approximating in size the protein molecule, e.g., certain substances present in supposedly protein-free filtrates of bacterial cultures. The specific carbohydrate complexes of pneumococci, which are not of themselves antigenic, give precipitin reactions with antipneumococcus serum.[107]

Neither the precipitin nor the antigen seems to be altered appreciably by the reaction, since when either is separated from the precipitate it retains its original properties. The amount of precipitation obtained is much modified by the amount of inorganic salts present, and, according to Friedemann,[108] there is a general resemblance between the precipitin reactions and the precipitations occurring when colloids precipitate one another, i.e., when an amphoteric colloid reacts with either an acid or a basic colloid.[109] The neutralization of the precipitinogen by the precipitin seems to follow the law of multiple proportions (Cromwell).[110] As there is no change observed in the surface tension during the precipitin reaction, it probably is not associated with any marked denaturation of the protein (Ramsdell).[111]

As with all the other "immune bodies" of this class, the precipitins have, in the Ehrlich nomenclature, a haptophore group by which they unite to the protein molecule, and another group by which they produce the change resulting in precipitation. When the latter group is destroyed by heating to 72°, the precipitin is converted into a *precipitoid,* which still possesses the property of combining with the antigen although without producing precipitation. Bacterial precipitinogens are relatively

[107] Felton and Bailey, *J. Infectious Diseases,* **38**:145 (1926).
[108] *Arch. Hyg.,* **55**:361 (1906).
[109] *See* Friedemann and Friedenthal, *Z. exp. Path. Therap.,* **3**:73 (1906); Iscovesco, *Compt. rend. Soc. Biol.,* 1906, vol. 61, and subsequent volumes.
[110] *J. Infectious Diseases,* **37**:321 (1925).
[111] *J. Exp. Med.,* **48**:615 (1928).

resistant to moderate heating, and heated extracts of bacteria are used for precipitin tests under the name *thermoprecipitins.*[112]

The Character of the Precipitate

It was first assumed that the precipitate was composed of the antigenic protein converted into an insoluble modification by the action of the precipitin, and the early literature referred to the antigen as the "precipitable substance," but it has been shown, especially by the work of Moll [113] and of Welsh and Chapman,[114] that the precipitate originates chiefly in the proteins of the immune serum. As the precipitate is said to be able to sensitize anaphylactically, both actively and passively, it would seem that it must contain both the antibody (which confers passive sensitization) and antigen (to cause active sensitization) (Weil).[115] Downs and Goodner [116] were able to demonstrate the presence of both the antigen and the precipitin in solutions of the precipitate made with weak sodium carbonate and then neutralized, also when the solvent was a concentrated solution of dextrose. The fact that the precipitate formed when diphtheria toxin is neutralized with antitoxin is able to incite antitoxin formation when used for immunization (Hartley) [117] proves the presence of the antigen in such precipitates.

The discrepancy between the amount of antigen and the amount of precipitate may be very great; for example, in one experiment of Welsh and Chapman, when 1 mg. of egg albumin reacted with a specific antiserum the precipitate weighed 25.9 mg. Just how much of the antigen is present in such precipitates does not seem to have been determined. As stated above, Weil found enough to sensitize guinea pigs to the antigen, and specific precipitins for both the antigen and the antibody were obtained by Fujiwara [118] on immunizing with the precipitate.

The amount of precipitate is always greater when the reaction is between homologous antiserum and antigen, than with even closely related but heterologous antigens,[119] so that the quantitative measurement of the precipitate is of value in applying this reaction to determine the nature of protein solutions. The dilution of the reacting solutions is of influence, however, for in too dilute solutions weak precipitins may

[112] *See* review by A. Ascoli, *Virchow's Arch.*, **213**:182 (1913).
[113] *Z. exp. Path. Therap.*, **3**:325 (1906).
[114] *Proc. Royal Soc.* (*London*) (**B**) **80**:161 (1908); *Z. Immunitäts.*, **9**:517 (1911).
[115] *J. Immunol.*, **1**:35 (1916).
[116] *J. Infect. Dis.*, **38**:240 (1926).
[117] *Brit. J. Exp. Path.*, **7**:55 (1926).
[118] *Mitt. Med. Fak. Univ. Kyushu*, **5**:325 (1921).
[119] Welsh and Chapman, *J. Hyg.*, **10**:177 (1910).

fail to give reactions; with strong precipitins the influence of dilution is much less. The precipitate, when of maximum amount, may contain more nitrogen than corresponds to the entire euglobulin of the immune serum, and the euglobulin apparently contains all the precipitin, so it seems probable that the precipitate consists of more than the precipitin alone.[120] According to Wu, Cheng, and Li[120a] the precipitate has a constant composition, and seems to contain no other proteins than those of the antigen and the antibody. The precipitate, at least when the antigen is whole serum, is insoluble in water, physiological salt solution, alcohol, weak acids and alkalies, resists trypsin, and contains about 25% of ether-soluble material, consisting chiefly of cholesterol and other lipoids (Hartley).[121] When fresh the precipitate may be redissolved at 55 to 56°, the solution being irreversible, and thus resembling lipoid-globulin precipitates.[122]

When strong antisera are placed in contact with the specific antigen in making precipitin reactions, a double ring of precipitate may form. According to Friedberger and Ikeda[123] the upper ring consists of lipoids; only the lower ring, which does not react for lipoids, contains antibodies that can unite with complement. Reiner and Kopp,[124] however, found that double rings occur in the absence of lipoids, and believe them to be merely the "Liesegang's rings" common to colloidal reacting mixtures.[124a] But the participation of the lipoids of the antiserum in the precipitin reaction is indicated by the observation of Hartley that ether extraction of the serum and the antigen renders them incapable of producing a specific precipitate, although ether extraction does not prevent toxin-antitoxin or agglutinin reactions. Apparently the lipoid has to do with the formation of the precipitate, of which it forms a large part, rather than with the reaction between the antigen and the antibody.

The Precipitin

Precipitin appears in the blood generally about six days after injection of the protein, but disappears after injection of each subsequent dose of protein, to reappear again after a somewhat shorter lapse of time. After injections are stopped, the precipitin disappears rather

[120] The precipitate is always less in amount than the total globulin of the antiserum [Franceschelli, *Arch. Hyg.*, **69**:207 (1907)].
[120a] *Proc. Soc. Exp. Biol. Med.* **25**:853 (1928).
[121] *Brit. J. Ex. Path.*, **6**:180 (1925).
[122] Ottensooser, *Z. Immunitäts.*, **43**:91 (1925).
[123] *Klin. Woch.*, **4**:2149 (1925); *Z. Immunitäts.*, **49**:481 (1925).
[124] *Kolloid Z.*, **42**:335 (1927).
[124a] Sometimes even three or four zones may appear, especially when there is an excess of antigen. [Goldsworthy, *J. Path. Bact.*, **31**:525 (1928).]

rapidly, but never appears in the urine, although it may enter the fetal blood from the blood of pregnant female animals. The presence of precipitins in the blood does not seem to prevent the excretion of the foreign protein in the urine. Precipitin and antigen have been said to coexist ununited in the circulating blood under certain conditions (see pp. 127-129), and the presence or absence of an abundance of precipitin in the blood of an animal does not markedly alter the disappearance of antigen injected into the blood of such an animal, according to Mackenzie.[125] From the above facts it might seem doubtful that the precipitin actually functions to protect the body from foreign proteins, as it has generally been assumed to do. But Opie[126] finds that in immunized animals antigenic material injected into the tissues is retained there, thus being prevented from entering the circulation and producing systemic effects; possibly precipitation by antibodies may have a part in this local fixation of antigen, which is followed by its destruction. Presumably if precipitation *in vivo* is followed by destruction of the precipitate this is accomplished either by phagocytic cells or by enzymes present free in the blood or tissue fluids.[127]

Certain antibodies are carried down with the precipitates formed in the precipitin reaction when the serum containing them reacts under proper conditions with an antiserum; e.g., diphtheria antitoxin is precipitated when added to the serum of a rabbit immunized to horse serum.[128] As the precipitates formed in the precipitin reaction, when injected into a guinea pig, make it passively hypersensitive to the protein used as antigen in the precipitin reaction, it would seem that the precipitin and the anaphylactic antibody are identical (Weil),[129] or at least closely associated. (For details see Chapter IX.)

The Mechanism of the Precipitin Reaction

As with agglutination, the H-ion concentration has no marked influence. Precipitin reactions are obtainable within a pH range of 4.5 to 9.5 and the precipitation is no heavier at the iso-electric point of either the serum globulin or the antigen; if the pH is outside this range no precipitate forms, and an existing precipitate will be redissolved by such H or OH concentrations (Mason).[130]

[125] *J. Exp. Med.*, 37:491 (1923).
[126] *J. Exp. Med.*, 39:659 (1924); *J. Immunol.*, 9:231 and 259 (1924).
[127] Paul Hirsch and Koehler [*Fermentforschung*, 6:56 (1922)] report that, according to the evidence furnished by the interferometer, the precipitin reaction occurring *in vitro* is accompanied by a fall in the total amount of protein and an increase in the amount of non-protein nitrogen in the solution, which suggests that a cleavage takes place.
[128] *See* Gay and Stone, *J. Immunol.*, 1:83 (1916).
[129] *J. Immunol.*, 1:35 (1916).
[130] *Bull. Johns Hopkins Hosp.*, 33:116 (1922).

It is, of course, perfectly possible to have a union of precipitin and antigen without any visible precipitate, since the product of the reaction is not necessarily insoluble under all conditions; in this case the occurrence of a reaction must be demonstrated by some other method, e.g., the complement fixation reaction, q.v.

The Zone Phenomenon

This is particularly well seen in precipitin reactions, for since both reacting substances are colloids they follow the laws governing other mutually precipitating colloids, and precipitation occurs only when they are brought together in concentrations that lie within definite zones of relative proportions. The formation of precipitates when precipitin acts on the antigen (precipitinogen) occurs according to the following rules (Fleischmann and Michaelis): [181]

(a) With a given quantity of precipitinogen the amount of precipitate increases with increase in the quantity of precipitin.

(b) With a given quantity of precipitin the amount of precipitate increases at first with addition of precipitinogen, then decreases, and with a certain excess of the latter approaches zero, this being the "zone phenomenon."

(c) With constant amounts of both, the quantity of precipitate decreases as the amount of fluid in which the reaction takes place increases.

The following table, taken from Zinsser, illustrates the zone phenomenon seen with an excess of antigen, which in this case is in excess when it consists of but 0.05 to 0.005 cc. of sheep serum.

Sheep serum 0.5 cc.		Antisheep rabbit serum	Precipitate
1:10	+	0.5 cc.	—
1:50	+	0.5 cc.	±
1:100	+	0.5 cc.	+ +
1:500	+	0.5 cc.	+ + +
1:1,000	+	0.5 cc.	+ +
1:5,000	+	0.5 cc.	+

Doerr [182] calls attention to the marked similarity in the behavior of precipitin reactions and the precipitation of proteins by salts of cerium and thorium, which are effective in very great dilutions, producing inert colloid precipitates which dissolve readily in an excess of either component.

The interference with or re-solution of precipitate seen in the zone

[181] *Biochem. Z.*, 3:425 (1907).
[182] *Kolloid Z.*, 27:277 (1920).

phenomenon is exhibited most strikingly when it is the antigen which is in excess; the amount of antibody present usually makes much less, if any, difference. This zone phenomenon is at least quantitatively specific, in that only the specific antigenic protein has the full inhibiting effect.[133] For example, in the reaction between isolated serum globulin and a specific antiserum for this globulin, the precipitate is more readily redissolved by the same serum globulin than by serum albumin from the same species or by serum globulin from another species (Demme).[134] When the inhibition is complete it cannot be overcome by further addition of antigen, but further addition of antiserum will do so (Welsh and Chapman).[135]

This solvent action of excess of antigen is seen not only with isolated serum proteins, as studied by Demme, but also by crystallized egg albumin [136] and even with the non-antigenic carbohydrate-like substance of pneumococci which gives specific precipitation reactions with antipneumococcus serum.[137]

Holker [138] describes the physical chemistry of the zone reaction as exhibited by precipitin sera and antigen, as follows: When only a minimal quantity of antigen is added to serum the very largest of the euglobulin particles are precipitated, and so the amount of the precipitate is small. With increasing quantities of antigen smaller particles are included, and so the amount of the precipitate increases. With a still further increase in the quantity of antigen the electric potential on the surface of the whole of the euglobulin is reduced to zero, and therefore the precipitate reaches a maximum in quantity. With increase of antigen beyond this point, the potential on some of the serum particles is carried beyond zero and reversed by the charge on the antigen which serves to stabilize these particles. Hence the precipitate becomes smaller in amount. This process steadily goes on with increasing doses of antigen, until at a certain strength all the euglobulin particles are stabilized by adsorption of antigen, and no precipitation occurs.

Physical Chemistry of Precipitin Reactions

E. F. Hirsch [139] has analyzed the physical chemistry of colloidal precipitation and the precipitin reaction as follows: "Proteins are re-

[133] *See also* Downs and Goodner, *J. Infectious Diseases,* **38**:240 (1926).
[134] *Cellule,* **24**:315 (1907).
[135] *J. Path. Bact.,* **13**:206 (1908-09).
[136] Opie, *J. Immunol.,* **8**:19 (1923).
[137] Morgan, *J. Immunol.,* **8**:449 (1923).
[138] *J. Path. Bact.,* **25**:281 (1922).
[139] *J. Infectious Diseases,* **30**:666 (1922).

garded, generally, as amphoteric electrolytes, that is, substances which are able to unite with acid as well as alkali. The hydrogen-ion concentration of the surrounding solution determines whether they unite with the one or the other. When the hydrogen-ion concentration of the solution exceeds a critical point which is known as the iso-electric point of the protein, the protein combines with acid to form a salt which, dissociating, gives rise to a protein cation carrying a positive electrical charge and an acid anion with a negative electrical charge. When the hydrogen-ion concentration of the solution is on the alkaline side of the iso-electric point of the protein, the protein combines with metals to form a salt such as sodium proteinate which dissociates into negatively charged protein ions, and positively charged ions of the metal.

"That precipitation of a colloid bearing an electrical charge of one sign is accomplished by the addition of a colloid bearing an opposite charge, and that both colloids are carried down in the precipitate, is generally recognized. The precipitin reaction on mixing an antigen in solution with its homologous immune serum probably belongs to the same group of reactions. Blitz suggests the following rules regarding the precipitation of one colloid by another when mixed together quickly and uniformly:

" 'If to a given colloidal solution, one of the opposite sign is added in small proportion, there is no precipitation. As the quantity of the second increases, the coagulative action follows parallel until a proportion is reached which causes immediate coagulation. As the amount is still further increased, coagulation ceases; that is, there is an optimum precipitation for certain proportions, and when these favorable proportions are exceeded on either side, no precipitation occurs.' "

Hirsch found that the precipitation of human serum by homologous immune serum is accompanied by an increase in the alkalinity of the medium similar to that observed on the agglutination of bacteria by immune serum and on the precipitation of colloidal gold by spinal fluid (Lange test). The increase of hydroxyl ions on the precipitation of human serum by homologous immune serum probably results from a liberation, from protein salts, of the base (Na) whose dissociation constant is greater than that of the immune substance.

Since the bacteria in a suspension in salt solution and the colloidal gold particles of the gold solution both carry negative electrical charges, it is likely that their agglutination or precipitation are similar chemical reactions, that the changes are governed by well-known laws obtaining in the precipitation of a colloid bearing an electrical charge

of one sign by the addition of a colloid with an opposite electrical charge, and that the precipitate contains both the precipitating and the precipitated colloids. The precipitin reaction is analogous, at least to a certain extent, with these reactions, which differ most conspicuously in their lack of any corresponding degree of specificity.

So far, however, attempts to interpret the precipitin reaction, as Arrhenius has tried to do, *solely* on the basis of the laws of physical chemistry, have not met with much success.[131] We prefer the attitude of Krogh,[140] who states that the colloidal chemical part of immunological reactions is to be looked upon as only a preliminary step to the real chemical process that completes the reaction and gives it the specific characters.[141]

Recapitulation

The agglutination and precipitation reactions are characterized by the reduction of the degree of dispersion of colloids either in suspension or in solution, so that the aggregated particles become visible, form coarse flocculi and precipitate. The maintenance of suspension of colloidal particles probably depends upon (1) the repellent forces of like electrical charges on the particles which prevent coalescence, and (2) the Brownian movement which prevents gravitation in very fine suspensions; whereas the aggregation of colloidal particles depends on the cohesive forces which draw the particles together. Therefore, the existence of a suspension, and equally the agglutination of its particles (whether these are molecules, micellæ or larger masses), depends upon the relative strength of these opposing forces. The agglutination and precipitation reactions produced by a specific immune serum acting upon a specific antigen, which is usually if not always a protein, are closely related to the corresponding reactions in colloidal chemistry. In many essential respects these immunological reactions are similar to, and perhaps identical with, the precipitation of colloids under the influence of other colloids of different electrical charges, or by electrolytes.

[140] *J. Infectious Diseases,* 19:452 (1916).

[141] An attempt to put the precipitin reaction on such a double colloidal and chemical basis is made by Paul Hirsch, who suggests that specific enzymes, the defensive ferments of Abderhalden (*q.v.*), break down the antigenic protein until the liberated amino acids increase the pH of the mixture to the isoelectric point of the serum globulin which is then precipitated. Langenstrass [*Fermentforschung,* 3:1 (1919)] believes that his measures of the change in the reacting mixtures by means of the refractometer support but do not establish this hypothesis. This was not confirmed by Doerr and Berger [*Biochem. Z.,* 123:144 (1921)]; and by Bachmann [*Z. Immunitäts.,* 35:462 (1923)] who could find no measurable change in refraction during the precipitin reaction. Furthermore, as Langenstrass admits, specific precipitin reactions occur in much more dilute solutions of globulin than can be made to precipitate merely by bringing the solution to the isoelectric point.

Precipitation and agglutination reactions cannot take place in the absence of salts, for although the antigen and antibody combine, it requires the presence of at least minute amounts of electrolytes to cause aggregation of the colloidal complex.[142] Evidently the reaction between the antibody and the antigen leads to the formation of a colloidal complex which is highly precipitable by electrolytes, and corresponds to the reaction observed when to a solution of a colloid is added another colloid of opposite charge in too small amount to induce flocculation, for in such a situation the addition of even a small amount of salt may then bring about flocculation. Apparently the antigen-antibody reaction leads to the formation of an electrically amphoteric colloidal suspension, so that the ions of electrolytes cause precipitation by discharging the particles.

The relation of these reactions to colloidal phenomena is also seen in the occurrence of the "zone phenomenon" characterized by the fact that an excess of one of the reacting colloids may reduce the amount of flocculation or even prevent it entirely.

As yet we cannot explain exactly how these colloidal reactions of immunology are brought about, and their striking specificity does not support the idea that the simple colloidal reactions alone are all that is involved. The specificity of the reactions suggests some more definitely chemical reaction, presumably superimposed on the colloidal reactions which undoubtedly take place. Until we know more than we now do concerning the colloidal reactions and their relations to chemical reactions in general, and until the underlying principles of solution are better understood, the details of these remarkably delicate and specific immunological reactions cannot well be explained.

Numerous suggestions have been made concerning the processes involved. For example, that agglutinins attack some protective colloid about the bacteria or other cells which are to be agglutinated, whereby they become like unprotected inorganic suspensions which are precipitated by salts and other electrolytes. Or that the antibody alters the antigen so that it is more capable of combining with or adsorbing salts, which then reduce the colloidal dispersion. Or that the antibody increases the cohesive force which brings the particles together and overcomes the repellent effect of the like electrical charges of the particles.

There is abundant evidence that the antibodies, and likewise the globulins of the immune serum, are deposited on the surface of the

[142] Except at the isolectric point.

sensitized cells, and that the resulting complex behaves like denatured proteins in being readily flocculated and precipitated by electrolytes.

As to details, the antigens in these as in all other reactions are generally and perhaps always proteins, although the possibility of the existence of non-protein colloidal antigens cannot be excluded. There is evidence that the antigen may sometimes be of smaller molecular dimensions than the antibody, at least some antigens diffuse more rapidly than the antibody as it exists in the serum. The reactions are accompanied by an increased OH concentration in the reaction mixture, perhaps through a discharge of Na from the antigen and of OH from the antibody during their reaction, which depends on the mutual neutralization of their electrical charges. However, the reactions between antigen and antibody take place within a wide range of pH, and are not appreciably greater at the pH of either the antigenic protein or of the serum globulins with which the antibodies are associated. The serum of normal animals will often give agglutinin and precipitin reactions in high concentrations (1-10) and it is not known whether these "normal antibodies" are identical with the antibodies which are developed on immunization.

In the precipitin reaction the bulk of the observed precipitate is derived from the proteins of the immune serum, and there is comparatively little of the antigen present in this precipitate, which seems to consist chiefly of serum globulin and lipoids and to possess the property of sensitizing guinea pigs passively to the same antigen as the one with which the precipitin reacts (passive anaphylaxis q.v.).

We do not yet know to what extent the precipitin and agglutination reactions protect the body against either bacteria or foreign proteins. It is possible that they have some influence, but they seem to be, at the best, of minor importance as compared with either the antitoxic neutralization of toxin or the cytolytic and proteolytic reactions.

CHAPTER VII

THE LYTIC REACTIONS

Bacteriolysis, Cytolysis, Amboceptor-complement Reactions

One of the earliest observations in the history of modern immunology was that blood has the power to destroy bacteria. In 1888 Nuttall found that normal defibrinated blood when incubated with tissue containing anthrax bacilli brought about a reduction in the number of bacilli. Buchner in 1889 found that this bactericidal power of the blood is destroyed by moderate heating (56° C. for 30 min.), pointed out the resemblance of the behavior of the active substance to the enzymes, and christened it alexin (protective substance), a name now more in use than formerly.[1] A few years later Pfeiffer observed that when cholera spirilla are injected into the peritoneal cavity of a guinea pig which has been immunized to these organisms, they lose their motility, break up in granules and finally disappear. He also found that serum from an immune animal if injected along with the spirilla would cause the same bacteriolysis to take place in a normal animal. Metchnikoff found that the same process could be brought about outside the animal body if the spirilla were exposed to the action of the immune serum and an extract of leucocytes, indicating that at least one element of the alexin might come from the leucocytes. Bordet found that leucocytes were not necessary to accomplish bacteriolysis, observing that a fresh immune serum, or a heated immune serum to which a small amount of normal serum had been added, will also accomplish bacteriolysis *in vitro*. These observations led to the conclusion that bacteriolysis depends upon the presence of two agents, one present in normal blood and readily destroyed by heating, called the *complement* because it complements the action of the other agent which is more heat-resistant, found in much greater abundance in immune than in normal blood,

[1] By alexin Buchner meant the entire bactericidal agent of the blood serum, which we now know consists of two elements, the specific immune body (amboceptor or sensitizer) and the non-specific normal constituent of serum which has the cytolytic effect when united to the antigenic cell by the antibody. Bordet gave the name alexin to this second agent, which Ehrlich called complement. Probably the doubt as to which is meant by the term alexin, whether Buchner's bactericidal agent or Ehrlich's complement, has caused the use of the name complement to be generally preferred.

and, as later observations showed, specific in its capacity to react selectively with the antigen used in immunizing. The next step in the development of this subject was the discovery that red corpuscles may similarly be dissolved by the action of a specific immune antibody (obtained by immunizing with red corpuscles) and the complement.[2] Later, cytolysis of other cells was found possible under similar conditions, demonstrating that bacteriolysis is only one instance of a process which may be capable of attacking any or all other cells.

Cytolysis

The destruction of cells by specific immune serum introduces a new element not seen in the reactions previously discussed. In agglutinin and precipitin reactions we see a reduction in the amount of dispersion of the colloids in the reaction mixture, but in cytolysis we may witness the reverse process, whereby the colloidal aggregates go into solution, even when they are such firm and complex masses as tissue cells or bacteria. This disintegration of cells is accomplished by a process that resembles the action of proteolytic enzymes and the mechanism of the reaction seems to be quite different from that of the neutralization of toxins by antitoxins, or the agglutination or precipitation of colloidal suspensions or solutions. That this lytic action depends upon the presence in the serum of the immunized animal of two distinct agents, separable because one is much more resistant to heat than the other, is demonstrated by such an experiment as the following:

If we heat bactericidal serum obtained by immunizing an animal against bacteria, say the cholera vibrio, at 55° for fifteen minutes, it will be found to have lost its power of destroying these organisms.[3] Normal serum of non-immunized animals is equally without effect upon the vibrios. If, however, we add to the inactivated heated serum an equal quantity of unheated normal serum, the mixture will be found to be as actively bactericidal as the original unheated immune serum. This phenomenon is interpreted to mean that, by immunization, some new substance has been developed which, although by itself incapable of destroying bacteria, is able, when united with some substance present in normal serum, to destroy bacteria readily. The substance present in normal serum is also incapable of affecting bacteria by itself, but needs

[2] For full review on hemolysis *see* Sachs and Klopstock, "Methoden der Haemolyseforschung." Berlin, Urban and Schwarzenberg, 1928.

[3] Normal *human* serum often exhibits some slight power to destroy bacteria, even after heating to 55°. The nature of this thermostable bactericidal agent is unknown. [*See* Selter, *Z. Hyg.*, 86:313 (1918).]

the presence of the substance developed by immunizing to render it bactericidal. Hence *the bactericidal property in this case depends on two substances acting together:* one, developed during immunization and therefore called the *immune body,* is specific for the variety of bacteria used in immunization, and is not destroyed by heating at 55°. The other, present in normal serum, is not increased during immunization, is not specific in character, and is destroyed by heating at 55°; as its action is complementary to that of the specific immune body, it is called the *complement* (and also, *alexin*).

It was believed by the Ehrlich school that the action of these substances is as follows: The immune body is, like antitoxin, a cell receptor which unites the bacteria to the cell. It differs from the antitoxin, however, in that it has two affinities, one for the complement and the other for the bacterial substance. On account of the existence of the two affinities it was called an *amboceptor.* Some serums contain such amboceptors for certain bacteria without previous immunization, hence the term *immune amboceptor* is reserved for amboceptors developed by immunization.

Bordet found it unnecessary to accept Ehrlich's views of the amboceptor character of the specific immune body, which assume the presence of one definite group uniting it to the complement and another to the antigen. He prefers to characterize the action of the immune body as that of a *sensitizer* which, when combined with the antigen, creates a complex which has an avidity for the complement. Experiments to be cited later have shown that only after the antigen has been acted upon by the immune body can the complement be bound by the latter, for in the absence of the antigen, complement and immune body may coexist in the serum without union. Bordet's views now seem to be much more closely related to the facts than the original Ehrlich hypothesis.

Properties of Amboceptors or Sensitizers

The function of the immune body is to unite the antigen to the complement, or, to put it in a more strictly chemical way, the addition of the amboceptor to the antigen gives it a chemical or physical affinity for complement. It is, therefore, an *intermediary body,* uniting the complement to the cellular protoplasm which constitutes the antigen. As indicated in Chapter IV, there is not a little reason to believe that the amboceptor is identical with the precipitins and the agglutinins,

manifesting itself only when the conditions are favorable for the demonstration of the lytic reaction, that is, when there is an abundance of complement and a type of cell which readily undergoes solution, e.g., red corpuscles, cholera spirilla.[4] Some cells which are not so readily dissolved, e.g., typhoid bacilli or spermatozoa, under these conditions may undergo agglutination into firm masses with relatively little surface exposed to attack and so the amount of cytolysis may be unobservable. However, the cells may be killed even if not dissolved, and then we speak of the immune serum as exercising a *bactericidal* or *cytotoxic* effect, rather than cytolysis which implies solution of the antigen.

Amboceptors have the usual characters of the immune bodies as far as can be determined in their unisolated condition. They are highly specific, but, on account of their great activity, exhibit strongly the group reactions for related antigens, especially when being tested against such complex antigens as cells and sera.

Gay and Moreschi found that the precipitates formed by the action of a specific immune serum upon the antigen, are able to unite with complement, so that evidently the precipitate in the precipitin reaction contains the amboceptor.[5] As it has also been shown (see p. 111) that the precipitate contains the precipitin, the inference is natural that the precipitin and the cytolytic amboceptor are one and the same immune body. As union of amboceptor and complement, or complement fixation (see p. 194), is a more delicate reaction than that of precipitation, the fact that a serum may show the former reaction and not the latter is no evidence that the two reactions depend on different antibodies. As Zinsser has stated, the visible precipitation is a secondary phenomenon, dependent upon the existence of suitable conditions which favor flocculation; the essential, primary phenomenon is the union of antigen and antibody which may be demonstrable by the complement fixation reaction even when no visible precipitation is present. In fact, the maximum fixation of complement occurs when the particles of precipitate are still so small that they do not cause turbidity in the reacting mixture.[6]

The stability of the amboceptors is very considerable; e.g., serum prepared in 1895 by Pfeiffer against cholera vibrios was found to have lost almost none of its activity after eight years in an ice-box (Fried-

[4] Bacteriolysis is usually much less marked in test tube experiments than in the living animal. [*See* di Renzo, *Z. Immunitäts.*, **38**:519 (1924).]

[5] When precipitin reacts with antigen in the form of a bactericidal serum, the resulting precipitate removes much of the bactericidal agent [Todd, *Brit. J. Exp. Path.*, **7**:368 (1926)].

[6] Goldsworthy, *J. Path Bact.*, **31**:220 (1928).

berger). Heating twenty hours at 60° scarcely injures them, but 70° for one hour destroys them almost completely, and heating the unmodified serum to 100° destroys all the immune bodies. Heat lability apparently depends on coagulation of the serum proteins, for addition of weak alkalies or other agents that prevent coagulation greatly decreases the amount of antibody destruction by heat up to 90°.[7] They are quite resistant to putrefaction, and, like the antitoxins, do not dialyze. Strong salt solutions will prevent the union of complement and amboceptor *in vitro,* and probably to greater or less degree in the animal body, but the union of antigen and amboceptor is not prevented by salt.[8] Alkalies may prevent the union of amboceptor with the cells, or extract it from the cell to which it has united; and they may also inhibit the union of amboceptor and complement. Amboceptors are not inactivated by shaking, as is complement, but they are destroyed alike by ultraviolet rays, and both resist x-rays.[9]

Pick, Rhodain, and Fuhrmann found that immune amboceptors are precipitated entirely in the euglobulin fraction of the serum protein. Other authors have reported finding them chiefly in the pseudoglobulin fraction, which contradiction merely indicates the lack of agreement as to the individual existence and separation of the serum proteins.[10] By means of electrodialysis, Locke and Hirsch [11] separated out hemolysin quantitatively as pseudoglobulin, which contained 150,000 to 200,000 units per gram. The hemolysin has been concentrated still more, after being concentrated on sensitized corpuscles and then splitting off in a relatively pure state.

According to Pfeiffer and Proskauer,[12] digestion of the globulin precipitate, in which amboceptors are carried down, does not destroy their activity completely even when all the proteins are thus removed, as far as ordinary protein tests indicate. Immune serum kept three months in alcohol yielded an extract with distilled water that was rich in immune bodies, but almost free from protein. From these experiments it has been thought by some that the bacteriolytic amboceptor is not itself a protein, although closely associated with the serum globulins. This view is supported by Huntoon, who isolated the bactericidal immune body from antipneumococcus serum in a state so free from proteins that no

[7] *See* Silber and Shaechter, *Z. Immunität.,* **53**:263 (1927).
[8] Angerer, *Z. Immunitäts.,* **4**:243 (1909).
[9] Scaffidi, *Biochem. Z.,* **69**:162 (1915).
[10] *See* Coulter, *J. Gen. Physiol.,* **10**:545 (1927).
[11] *J. Infectious Diseases,* **35**:519 (1924); **37**:449 (1925).
[12] *Centr. Bakt.,* **19**:191 (1896).

chemical evidence of protein could be found in solutions that were nevertheless strongly bactericidal. (See page 124 for details.)

In favor of the protein character of amboceptors, however, is the fact that they are antigenic, for anti-amboceptors may be obtained by immunizing an animal with a serum which contains amboceptors. Furthermore, an antiserum obtained by immunizing an animal with normal serum from a given species will inhibit the amboceptor action of an immune serum from any animal of that species, e.g., the antiserum against rabbit serum proteins obtained by immunizing a sheep with rabbit serum, will inhibit the amboceptor action against cholera spirilla of the serum of a rabbit immunized to cholera spirilla. That is to say, antibodies against rabbit serum proteins are also antibodies for rabbit serum amboceptors, supporting the view that these amboceptors are serum proteins and not something new introduced solely during immunization. There is also the observation of Mudd [13] that sensitized cells behave as if they had become coated with a film of protein; and Locke and Hirsch [14] have found that the most highly purified hemolysins, obtained by splitting the antibody from sensitized corpuscles contains protein.[15]

Landsteiner and Jagic [16] found that red corpuscles treated with silicic acid under proper conditions are sensitized to the hemolytic action of complement in much the same way as corpuscles sensitized with immune amboceptor, except that the sensitization is not specific, for many sorts of corpuscles may be sensitized by the silicic acid. Hence this observation does not explain the action of immune amboceptors, and it does not tell us what the amboceptor does to the sensitized antigen. According to Singer [17] the union of amboceptor (hemolytic) with its specific antigen follows the law of adsorption.

With all the evidence available as to the "unity of antibodies" there is a growing tendency to question the very existence of antibodies as concrete substances, and to explain all the properties of immune sera as the result and expression of changes in the lability or aggregation

[13] *J. Exp. Med.*, **43**:127 (1926).
[14] *J. Infectious Diseases*, **37**:449 (1925).
[15] Uchida [*J. Infectious Diseases*, **40**:588 (1927)] found purified hemolysin to be a non-lipoidal colloid but did not establish or disprove its protein character. F. Klopstock [*Deut. med. Woch.*, **51**:592 (1925)] has supported the idea that in the case of specific hemolysis by immune sera, and presumably in other lytic reactions, the hemolysins are derived from disintegration products of the injected cells, which combine with pseudoglobulin of the serum to form a complex which exhibits enzyme functions; the specificity depends on the origin of these hemolysins from the injected corpuscles.
[16] Landsteiner and Jagic, *Wien. klin. Wochschr.*, **17**:63 (1904); *Münch. med. Wochschr.*, **51**:1185 (1904).
[17] *Z. Immunitäts.*, **35**:191 (1922).

of the serum proteins.[18] F. Klopstock [19] says that instead of thinking of a receptor apparatus, one should consider that antibodies merely place an antigen in a specific colloidal state in which it may be acted upon by an enzyme.

Properties of Complement or Alexin

The complement is the substance that actually destroys the sensitized cells, in which respect, as well as in its susceptibility to heat, it resembles the enzymes. Complement is present in normal serums, and, as it is not increased in amount during immunization, it may not be sufficient to satisfy all the amboceptors, hence it may be impossible to secure marked bactericidal effects with an immune serum even when an abundance of amboceptors is present. If the complement in an immune serum has been destroyed by heating, it may be replaced by adding normal serum from another animal, even of some other species, indicating that the complement is not specific in its nature, and that quite the same complement may be present in the blood of many different animals.

It having been found that a single normal serum is capable of functioning as complement with any variety of specific amboceptors to bring about lytic reactions with various sorts of antigens (e.g., red corpuscles, bacteria), it is assumed that there is but one sort of complement which is capable of uniting with many different sorts of amboceptors. Furthermore, the complement of one sort of serum may unite with amboceptors in inactivated immune sera from many different species. For example, normal guinea pig serum may be used to furnish the complement for a reaction between goat serum and beef corpuscles, or between rabbit serum and typhoid bacilli, and will participate in the reaction equally well in either case. There are, however, quantitative differences in the activity of complement from different species, and a serum which may be actively complementary in some reactions may be relatively inactive in other combinations. These differences presumably depend on the conditions within the fluid in which the reaction is occurring, and especially the character of the other colloids present, for these may readily behave as protective colloids and interfere with reactions.[20] In view of the fact that normal guinea pig serum may function as complement in reactions with as many different sorts of specific sensitizing antibodies as we care to prepare by immunizing with

[18] *See* Silber and Tschernochwostow, *Z. Immunitäts.*, **48**:472 (1926).
[19] *Centr. Bakt.*, **100**:90 (1926).
[20] Mackie found that complement in serum from different species may differ in respect to the proteins with which it is associated. [*J. Immunol.*, **5**:379 (1920).]

different antigens, it seems much more probable that there is one common complement normally present in serum capable of reacting with any and all sorts of immune antibodies, than that there are as many different complements preformed in normal serum as there are possible antigens.

The origin of the complement is unknown, but it has been urged that the leucocytes are an important source of this substance. There is evidence, however, that various organs and cells may also produce complement;[21] and also evidence that leucocytes are not its source.[22] Its most important characteristics are its extreme susceptibility to heat and other physical agents, and the resemblance of its action to the action of enzymes.[23] Hektoen[24] found that it could be made to unite with Mg, Ca, Ba, Sr, and SO_4 ions, which rendered the complement (for typhoid bacilli and red corpuscles) inactive.[25] Manwaring,[26] found that these ions could be separated again from the complement by simple chemical precipitation. Acids stronger than CO_2 and of the higher saturated or unsaturated fatty acid series, inactivate complement in strengths greater than n/40, and alkalies are equally inhibitive.[27] Addition of acids inactivates complement whenever the pH concentration passes the isoelectric point (Brooks).[28] Ultraviolet rays[29] and rays of the visible spectrum[30] destroy complement. Sherwood[31] has made a study of various substances that may be present in the blood in excessive amounts during pathological conditions, such as CO_2, lactic acid, acetone, etc., and finds that they interfere seriously with the action of complement.

The inactivation of complement at 56° is not entirely irreversible, for on standing there is a partial reactivation (Gramenitski).[32] Heat inactivation of serum is accompanied by a fall in surface tension, presumably depending upon an aggregation of the colloids in the serum with consequent diminution of surface tension, and such a change as

[21] *See* Dick, *J. Infectious Diseases,* **12**:111 (1913); and Lippmann and Plesch, *Z. Immunitäts.,* **17**:548 (1913).

[22] Morrison, *J. Immunol.,* **7**:435 (1922). Cholera antiserum will produce the Pfeiffer phenomenon of lysis of cholera vibrios in animals made leucocyte-free with thorium. [Lippmann, *Z. Immunitäts.,* **24**:107 (1915).]

[23] *See* Walker, *J. Physiol.,* **33**:p. xxi (1906).

[24] *Trans. Chicago Path. Soc.,* **5**:303 (1901-02). *See also* Tokunaga, *Centr. Bakt.,* **107**:283 (1928).

[25] Gordon *et al.* [*Biochem. J.,* **20**:1036 (1926)] state that nondiffusible calcium is concerned in complement activity.

[26] *J. Infectious Diseases,* **1**:112 (1904).

[27] Noguchi, *Biochem. Z.,* **6**:172 (1907).

[28] *J. Gen. Physiol.,* **3**:185 (1921).

[29] Courmont *et al., Compt. rend. soc. biol.,* **74**:1152 (1913); Gordon and Wormall, *Biochem. Z.,* **22**:909 (1928).

[30] Ecker, *J. Infectious Diseases,* **31**:356 (1922).

[31] *J. Infectious Diseases,* **20**:185 (1917).

[32] *Biochem. Z.,* **38**:504 (1912).

this in the complement may be what occurs in heat inactivation. In this respect, then, as well as in its inactivation by shaking, the complement behaves like a typical hydrophilic colloid which may be made to aggregate by physical forces, and if the process is not carried too far, more or less spontaneous dispersion may restore the colloid to its original state.

Presumably the complement is a protein,[33] for it has antigenic properties, so that immunization with sera containing either complement or complementoid causes anticomplement activity in the blood of the immune animal. Also, it is destroyed by trypsin free from lipase,[34] which attacks first the end piece or albumin fraction.[35] Like other colloids, complement is readily adsorbed by surfaces; like enzymes, it is destroyed by shaking, and it gradually disappears on standing.[36] Its colloid nature is attested by the large loss when complement is filtered through Berkefeld filters,[37] as well as by its behavior and properties in other respects.[38]

Apparently the complement molecule is larger than that of the immune bodies, for in the early stages of filtration through a Berkefeld filter, complement is held back while the immune bodies pass through (Muir and Browning).[39] Inactivation by salt (5 per cent) permits passage of the complement, and on dilution the activity is restored. If immune amboceptor and complement are mixed together at 37° and filtered, the amboceptor passes through and the complement is held back, showing that in the absence of antigen the amboceptor and complement are not united. There are some striking resemblances between the behavior of complement and of certain compounds of protein with soaps and lipoids, as pointed out especially by Noguchi,[40] but that these are identical with true bactericidal complement is doubtful, although the study of the complement in the Wassermann reaction (q.v.) has led to similar suggestions.[41] Thus, Liebermann has found that artificial

[33] An argument against the protein nature of complement has been advanced by Brooks [*J. Gen. Physiol.*, **3**:185 (1920)] based on a study of hemolytic sera, for he found that complement exposed to ultraviolet light is not thereby sensitized to heat as is the case with proteins. He advances the hypothesis that there is present in serum a hemolytic substance which is formed from a precursor which may resemble lecithin, and is constantly being formed and simultaneously being broken down into inactive products, but the activity of this lytic substance is dependent on the state of the serum colloids.

[34] Michaelis and Skwirsky, *Z. Immunitäts.*, **7**:497 (1910).

[35] Wormall, Whitehead and Gordon, *J. Immunol.*, **10**:587 (1925).

[36] Noguchi and Bronfenbrenner, *J. Exp. Med.*, **13**:229 (1911); Ritz, *Z. Immunitäts.*, **15**:145 (1912).

[37] *See* Schmidt, *Arch. Hyg.*, **76**:284 (1912); *J. Hyg.*, **14**:437 (1914).

[38] *See* Klopstock, *Deut. med. Wochschr.*, **50**:1790 (1924).

[39] *J. Path. Bact.*, **13**:232 (1909).

[40] Noguchi, "Serum Diagnosis of Syphilis and Luetin Reaction," Lippincott, Philadelphia, 1912.

[41] *See* Gordon *et al.*, *Biochem. J.* **20**:1036 (1926).

mixtures of methyl alcohol, sodium oleate, and calcium chloride, may be made to function like complement in dissolving sensitized corpuscles.[42]

Resemblance of Complement to Enzymes

A careful review of the evidence led Liefmann [43] to the conclusion that the reaction of complement to sensitized corpuscles is more like that of ferment to substrate than of antigen to antibody. In its effect of dissolving bacteria (and also other cells against which animals may have been immunized) complement resembles the enzymes, and by many it is looked upon as related to them, but the changes it produces do not resemble those produced by proteolytic enzymes in all details, although the curve of complement action resembles that of enzyme action.[44] In particular, complement seems to participate in reactions according to the law of definite proportions, unlike the enzymes.[45] In certain immune reactions, colloids (lecithin, silicic acid) [46] can play the rôle of complement and immune body, but these reactions are probably quite different from those of bacteriolysis by immune serum.

When hemolysis results from the action of immune hemolytic amboceptor and complement on red cells, the stroma of the corpuscles seems to remain intact and the hemoglobin is not demonstrably disintegrated, which facts speak against the proteolytic or enzymatic nature of complement action. Nolf was unable to find peptones in solutions in which red corpuscles had been hemolyzed by immune serum. Landsteiner and Lampl submitted to complement action the precipitate formed by the action of antiserum on horse serum, and could detect no loss of weight in the precipitate thus treated. Browning,[47] and Wollman and Graves,[48] also were unable to demonstrate that any proteolysis takes place during hemolysis by hemolytic serum.

On the other hand, when we use delicate methods to seek for the products of proteolysis in mixtures in which an immune serum has acted upon antigen, we can usually find them. This is the principle of the Abderhalden reaction (see p. 203) and demonstrates that proteolysis does occur in such mixtures. However, the digestion seems to attack the proteins of the immune serum rather than those of the antigen, and hence this demonstration of proteolysis offers no evidence that the

[42] *See* Born, *Z. Immunitäts.*, **44**:33 (1925).
[43] *Z. Immunitäts.*, **16**:503 (1913).
[44] Thiele and Embleton, *J. Path. Bact.*, **19**:372 (1915).
[45] *See* Liebermann, *Deut. med. Wochschr.*, **32**:249 (1906).
[46] Landsteiner and Jagic, *Wien. klin. Wochschr.*, **17**:63 (1904); *Münch. med. Woch.*, **51**: 1185 (1904).
[47] *Brit. Med. J.*, **1915**, Feb. 6, 242.
[48] *Compt. rend. acad. sci.*, **177**:1162 (1923).

destruction of cells by complement-antibody reactions depends on a proteolytic action of the complement on the antigen.

During lytic reactions the complement seems to be used up, and to participate in reactions according to the law of definite proportions,[45] in which respects it differs from a typical enzyme. Liefmann and Cohn [49] believe that the disappearance of the complement in these reaction mixtures does not depend on a quantitative union with the corpuscles, but on secondary processes which lead to its inactivation, and hence the disappearance of complement is not out of harmony with the possibility that it is an enzyme. Such secondary processes have been found to play an important rôle in the inactivation of enzymes, whereby their reactions come nearer corresponding to the laws of mass action than the theories of enzyme action predict. Thus, Northrop [50] found that pepsin combines with the products of hydrolysis and maintains an equilibrium with these according to the laws of mass action, only the uncombined residual pepsin continuing to hydrolyze the substrate. In such a proteolysis the pepsin is used up, just as complement is used up in cytolysis.

Structure of Complement

According to the Ehrlich theory, complement, like toxins and enzymes, possesses at least two groups: one, the haptophore, by which it unites with the amboceptor; the other, the toxophore (or *zymophore,* because of its enzyme-like action), which attacks the antigen. It may degenerate and lose its toxophore group while retaining the power to combine by means of its haptophore group, thus forming a *complementoid.* Complement and amboceptor can exist side by side in the serum, not uniting with one another until the amboceptor has become attached to the specific antigen.

It is stated that if serum which contains complement be so treated as to separate the globulins from the albumin, it is found that the complement has been divided into two parts, one present in each of the protein fractions. The globulin fraction of the complement will unite to amboceptor which is fixed to cells and hence is called the *mid-piece* of the complement, for it will unite also with the *end-piece* of the complement contained in the albumin fraction, and then cytolysis can take place. Without the intervention of the globulin mid-piece the albumin end-piece cannot unite with the amboceptor, while in the

[49] *Z. Immunitäts.,* 8:58 (1911).
[50] *J. Gen. Physiol.,* 2:471 (1920); 4:227 (1922).

absence of end-piece the amboceptor mid-piece complex can cause no cytolysis. Both fractions of the complement are destroyed by heat, but if the mid-piece is bound to the amboceptor it resists heating. The mid-piece corresponds to Ehrlich's haptophore, the end-piece to the toxophore group, and this complex structure is common to both bacteriolytic and hemolytic complement. The mid-piece seems in turn to be composed of two components, both globulins, one of which is more readily altered by heat than the other. A third component, characterized by being adsorbed by yeast, and of unknown structure, has been described,[51] and even a fourth unknown component has been suggested.[52]

Coulter [53] found that the destruction which complement undergoes on being heated in dilution in distilled water is least at a reaction between pH 6.1 and 6.4. This depends upon the relative preservation of the mid-piece function at this point. This reaction represents probably the isoelectric point of a compound of the euglobulin with some substance present also in serum. During the process of thermo-inactivation it is chiefly or entirely the ions of this euglobulin compound which react, and these combine or interact with substances contained in the pseudoglobulin and albumin fraction. The behavior of the euglobulin is different in the anionic and in the cationic condition, since on the acid side of pH 6.1 to 6.4 the destruction by heat increases with the acidity as rapidly in the presence as in the absence of NaCl. On the alkaline side of this point the presence of NaCl protects complement from destruction because of the depression in the ionization of the euglobulin.

Apparently, in view of what has been learned of the complex structure and the behavior of the complement, it would seem that there is ground to question even its existence as a definite substance or agent More probably what has been called complement merely represents a certain stage of aggregation and lability of the serum colloids, especially of the globulins.[54]

Complement Fixation (Bordet-Gengou Reaction) [55]

This much-used immunological reaction is based upon the following facts:

(1) Amboceptor (or sensitizin) and complement (or alexin) cannot unite with each other until the amboceptor (or sensitizin) has first

[51] Review by Parsons, *J. Immunol.*, 12:47 (1926).
[52] Whitehead, Gordon and Wormall, *J. Immunol.*, 13:439 (1927).
[53] *J. Gen. Physiol.*, 3:771 (1921).
[54] *See* Dean [*Lancet*, Jan. 13, 1917, p. 45); *also* Sachs, *Biochem. Z.*, 180:288 (1927)].
[55] For an extended study of the quantitative behavior of this reaction *see* Lasseur and Spillmann, "Réactions Anticorps. Etude Quantitative de la Fixation de l'Alexine," Imprimeries Réunies de Nancy, 1921.

been bound to the antigen, i.e., complement unites only with a sensitized antigen.

(2) The amboceptor is bound only by the specific antigen.

(3) After the specific amboceptor and antigen have once united, the resulting compound, i.e., sensitized antigen, will then unite with any complement which may be present, the complement not being specific. Therefore,

(4) The binding of complement is proof that a specific union of antigen and antibody is present in the materials under observation, because only such a specifically sensitized antigen can unite with complement.

(5) The presence or absence of free complement in the reaction mixture can be detected by exposing it to red corpuscles which have been sensitized (by treating them with serum containing specific hemolytic amboceptors but deprived of complement by heating at 55°). If free complement is present the sensitized red cells will undergo hemolysis, and conversely, absence of hemolysis will be proof that the complement has been previously bound by united antigen and amboceptor.

(6) Therefore, as the absence of hemolysis in such a system is evidence that in this system free complement has been exhausted because the specific antigen and amboceptor have both been present, by having either a known antigen or a known antibody we can demonstrate the presence or absence of the other.

That is to say, when sufficient amounts of mutually specific amboceptor and antigen are present the entire quantity of available complement will be fixed, and, consequently, the mixture contains no more complement available for further reactions. As complement does not ordinarily unite with amboceptors except when the amboceptors are united with their specific antigens, the fact that in a given system of

$$\text{complement} + \text{amboceptor} + \text{antigen}$$

there is no free complement is evidence of a reaction between amboceptor and antigen. In consequence of which this reaction can be used to determine the presence of a specific amboceptor in a serum, by using the corresponding antigen; or, conversely, with a serum containing a known amboceptor we can detect the presence in a solution of the specific antigen.

The indicator of the presence or absence of complement in universal use is the ability of the mixture to hemolyze red corpuscles that have

been sensitized by the specific hemolytic amboceptor. Thus, if typhoid bacilli and a typhoid antiserum which contains both complement and specific amboceptor, are mixed in proper proportions and incubated for a short time, the complement will be bound to the sensitized bacilli. If we then add this mixture to sheep corpuscles which have been acted upon by antiserum for sheep corpuscles from which the complement had been previously removed by heating, no hemolysis will occur, for we have added no *free* complement. But if our original mixture had contained dysentery bacilli instead of typhoid bacilli the complement would not have been fixed, and the addition to the sensitized sheep corpuscles of this mixture, which contains free complement, would cause prompt hemolysis.

Scope of the Complement Fixation Reaction

The principle was first worked out by Bordet and Gengou, after whom it is commonly called, with the use of bacteria and antibacterial sera. It at once gave a valuable method for identifying bacteria, by observing whether they do or do not bind the immune body (amboceptor or sensitizer) which is present in the serum of an animal immunized to known bacteria. Conversely, using known strains of bacteria it became possible to determine the presence or absence of specific antibodies in a given serum, which at times constitutes valuable evidence as to infection with these bacteria, quite analogous to the agglutinin reaction but far more delicate and of more universal application.

On account of the delicacy of this reaction it can be used to determine the presence in tissues of specific organisms which cannot be cultivated; thus, it was possible long ago to demonstrate the existence of a specific scarlatinal virus [56] in the tissues during this disease, although the actual organism had not been isolated. This fact led Wassermann to use extracts of the livers of congenital syphilitic fetuses, which contain great quantities of spirochetes, as an antigen for complement fixation reactions, whereby it should be possible to determine in a given serum the presence of specific amboceptors for the virus of syphilis, such amboceptors being present in persons infected with syphilis as a result of the reaction to the infection. As originally introduced, then, the Wassermann reaction was supposed to be simply a specific reaction between syphilitic antigen, specific syphilitic amboceptors, and non-specific complement. It was soon learned, however, that the reaction as it occurred in syphilis was decidedly different from the

[56] Koessler and Koessler, *J. Infectious Diseases*, 9:366 (1911).

original complement fixation reaction of Bordet and Gengou, for it was found possible to substitute in the reaction for extracts of tissues containing syphilitic virus (spirochetes), the most varied sorts of tissue extracts, coming from tissues certainly free from spirochetes (e.g., ox heart). This topic will receive further discussion later (Chapter VIII).

The complement fixation reaction does not require cellular antigens such as bacteria, which were used at first, but it depends on a general principle and is given with the greatest accuracy and delicacy of all the immunity reactions with any sort of antigen, whether in solution or in the form of cells. This at once brought the complement binding amboceptors into the same class as the precipitins and the agglutinins, and as is pointed out in Chapter IV, there is much reason to believe that these three reactions all depend on one and the same antibody, the differences merely being in the method of demonstrating the fact that such an antibody has reacted with a specific antigen.

Relation to Other Reactions

Gay [57] found that the precipitate which forms when a specific immune serum reacts with a dissolved antigen contains the complement-binding antibody, i.e., amboceptors or sensitizins. As it has been shown that this precipitate also contains the precipitin, we have here added reason to believe that precipitins and amboceptors are identical. Against the view of the identity of precipitins and sensitizing amboceptors has been advanced the fact that an immune serum may exhibit one of these reactions much more strongly than the other, and sometimes one may be demonstrable without the other, the complement fixation usually being the prevailing demonstrable reaction. This, however, loses its significance in the light of the fact that the conditions best adapted to bring out one reaction are usually not best adapted for the other, as Dean especially has pointed out (see p. 112). Furthermore, as explained elsewhere, the precipitin reaction requires rather nicely adjusted proportions of antigen and antibody for its demonstration, an excess of antigen inhibiting the formation of a precipitate as in the classical zone reactions of colloidal chemistry. But as the fixation of complement takes place in such a mixture even when no visible precipitate is present, the antibody may be demonstrated by this reaction when not shown by the precipitin test. This is illustrated by Zinsser in the following table, which compares the two reactions with the same antigen and immune serum.[58]

[57] *Centr. Bakt.*, 39:603 (1905).
[58] Zinsser, "Infection and Resistance," Macmillan, 1923, p. 209.

Sheep serum + antisheep serum		Precipitate	Fixation of 0.5 cc. guinea pig complement
0.5 cc. (1:20)	+ 0.5 cc.	+	Complete
0.5 cc. (1:50)	0.5 cc.	+ +	Complete
0.5 cc. (1:100)	0.5 cc.	+ + +	Complete
0.5 cc. (1:200)	0.5 cc.	+ + +	Complete
0.5 cc. (1:500)	0.5 cc.	+ + +	Complete
0.5 cc. (1:1,000)	0.5 cc.	+	Complete
0.5 cc. (1:2,000)	0.5 cc.	+	Complete
0.5 cc. (1:5,000)	0.5 cc.	—	Partial
0.5 cc. (1:10,000)	0.5 cc.	—	Partial
0.5 cc. (1:20,000)	0.5 cc.	—	None

The "zone phenomenon" (see p. 157) is shown to some extent in complement fixation as in other immunological reactions. If an excess of either antigen or antiserum is used the reaction may be prevented. To detect very small amounts of antigen, correspondingly small amounts of antiserum must be used (Parker),[59] and to get the greatest possible fixation the complement must be present when antigen and antibody come together (Dean).[60]

When there is an excess of antigen no precipitate is formed on the addition of relatively small amounts of precipitin, and when a precipitate has been formed by the action of precipitin on suitable amounts of antigen it may be redissolved to a greater or less extent by adding an excess of antigen. In either case, the clear fluid is capable of binding complement, showing that the antigen and antibody are present and united despite the absence of any visible precipitate. Dean [61] points out that the proportions of antigen and antibody which produce the largest quantity of precipitate are not those which effect the greatest fixation of complement. When antigen and antibody are mixed in the proportions most favorable for precipitation, the aggregation process takes place energetically and large flocculi are rapidly separated. But if the mixture contains a relatively smaller amount of antigen, precipitation is slower and less complete. These are the conditions favorable to complement fixation. Under these conditions the individual particles which form the precipitate are extremely small, and present in the aggregate an enormously larger surface area than the flocculi of a coarser precipitate. It seems probable that there is a direct relationship between the surface area of the particles of the precipitate and the amount of complement fixed.

"By taking any one antigen and any one antiserum and by varying

[59] *J. Immunol.*, 8:223 (1923).
[60] *Z. Immunitäts.*, 13:84 (1912).
[61] *Lancet*, Jan. 13, 1917, p. 45.

the relative proportions of these two ingredients it is possible to prepare mixtures which demonstrate either precipitation without complement fixation or complement fixation without visible precipitation. The amount of complement fixed is conditioned by two factors: (1) *The amount of precipitate formed* and (2) *the rate of precipitate formation.* Complement is fixed during the very earliest stages of the aggregation process which forms the precipitate. Indeed, after the process has advanced to a visible turbidity very little complement is taken up.[62] The relative proportions of antigen and antibody which favor rapid and complete precipitation are positively unfavorable to complement fixation. If a constant amount of antiserum be taken it will be found, as a rule, that the quantity of antigen which will produce the best complement fixation is many times less than the amount necessary to produce the largest precipitate. The reason why the two reactions do not run a parallel course is not that they are caused by two different sets of antibodies, precipitins and amboceptors, but because they represent two phases or two stages of the same reaction, and it may not be possible to demonstrate both stages under the same conditions. A flocculent precipitate represents the complete and final stage of a change which can be recognized in its earliest and incomplete stage by means of the complement-fixation method. On the other hand, if the mixture contains a very small amount of the antigen the aggregation process is extremely slow and may never proceed to the point at which even opalescence is produced. Such a condition is, however, very favorable to complement fixation."

Zinsser's views differ from this only in that he believes that the precipitation is merely a secondary, colloidal phenomenon, which may or may not coincide with the phase of greatest complement fixation, according to other fortuitous conditions which may favor or retard flocculation. Indeed, rapid compact precipitation may possibly be assumed to interfere with fixation in that it would inhibit perfect contact of the complement with the antigen-antibody complexes. Certainly fixation of complement does not depend on the production of a *nonspecific* precipitate in the serum, for Browning and Wilson [63] observed that if globin is added to any serum a precipitate is obtained which does not fix complement except in the case that the serum is an antiglobin immune serum, and then complement fixation takes place.

[62] *See* Goldsworthy, *J. Path. Bact.*, 31:220 (1928).
[63] *Brit. Med. J.*, 1:239 (1915).

The Physical Chemistry of the Complement Fixation Reaction

As with other antigen-antibody reactions, the amboceptor-antigen complex union is more or less reversible,[64] and this may be greatly affected by the pH of the fluid. Coulter [65] found that the combination of amboceptor (hemolytic) and antigen (erythrocytes) is related fundamentally to the isoelectric point of the serum globulin which carries the amboceptor, which seems to be between pH 5.2 — 5.4. In a salt -free medium the maximum combination is observed at pH 5.3, at which point the addition of electrolytes has no influence, although at reactions on either side of pH 5.3 the presence of electrolytes increases the proportion of combined antigen-antibody; i.e., electrolytes decrease dissociation of antigen and antibody at other reactions than the isoelectric point. Coulter says: "The amphoteric electrolytes, with which the immune bodies must be classed on the basis of their behavior in the electric field (Michaelis and Davidson; Landsteiner and Pauli), owe their electrical charge to ionization. On the alkaline side of the isoelectric point they ionize as acids, on the acid side as bases; at the isoelectric point the ionization is at a minimum. It is evident that the combination of sensitizer and cells is related intimately to the ionization of the immune body. The curves showing the fraction of sensitizer free in solution in a salt-free medium follow closely the curves given by Sörenson, after Michaelis, to represent the degree of ionization of an amphoteric electrolyte. The ionized fraction of the sensitizer, both as anion and as cation, corresponds with that fraction which is uncombined with cells, so that we may conclude that the cells combine only with the undissociated molecules of sensitizer."

Hecht [66] says we should look upon the property of serum which we designate as complement, as merely the property of a mixture of colloids and electrolytes in water, and dependent on such factors as the surface tension, viscosity, conductivity and dispersion. The complement function is manifested only when the colloid particles are of a certain size and electrolytes are present, and hence it is easily inactivated by changes in either the amount of electrolytes or the degree of dispersion of the colloids. Slight changes, therefore, produce a reversible inactivation; marked changes inactivate it irreversibly. All procedures that inactivate complement are associated with a reduction in surface tension. Conductivity is also lowered during inactivation except, of

[64] *See* Lasseur and Spillmann.[65]
[65] *J. Gen. Physiol.*, 3:513 (1921).
[66] *Z. Immunitäts.*, 36:321 (1923).

course, when this is accomplished by strong salt concentrations. These changes are not the result of alterations in the complement itself, but merely an expression of the change in the entire colloid complex of the serum. Anything which alters any of the colloidal functions of the serum will reduce its complement function, e.g., lowering of surface tension by freezing, or disturbing the conductivity by alcohol, distilled water, etc. The inactivation of complement by shaking is, as Hans Schmidt [67] has shown, not the result of chemical change, but merely the result of an alteration of the colloidal state of the serum which determines complement function.

Hecht [66] recounts in detail the agencies that are known to reduce complement activity and finds that this effect may always be explained by such changes as are described above. He points out that complement function occurs only when there is the combined action of several factors of which globulin-colloid (mid-piece), albumin plus lipoid colloid (end-piece) and electrolytes, constitute the best known combination. Change in the physico-chemical state of any one of these factors inactivates complement as a whole. From the foregoing considerations it seems probable that the smallest possible unit which exhibits complement function must consist of multiple protein molecules, which explains the pronounced colloidal character and ready inactivation of complement. In support of these views is the production of an artificial complement by Liebermann,[68] consisting of a combination of sodium oleate, proteins (including especially globulins), and calcium salts, forming an emulsion in a colloidal condition. This artificial complement is inactivated by heat, like natural complement, and can be used as complement in the Wassermann reaction.

Kiss [69] believes that the complement can be shown to be a definite body in the serum, consisting of most minute hydrophilic colloidal particles, which cannot be subdivided without loss of their characteristic properties. He found that all forms of complement fixation, whether specific or non-specific, follow the formula of physical adsorption, and hence must be looked upon as a physico-chemical process, but how the specificity of the reaction is to be explained on a simple physical basis has not been shown. And yet in none of the immunological reactions does the colloidal chemistry aspect stand out more prominently than in that of complement fixation. The ready inactivation of complement through adsorption by other colloids is a conspicuous feature of

[67] *Z. Hyg.*, 88:495 (1919).
[68] *Deut. med. Wochschr.*, 47:1283 (1921).
[69] *Biochem. Z.*, 129:487 (1922).

the behavior of this large colloidal complex, for silicic acid, gum mastic suspensions, and many other colloidal aggregates have this effect quite as strikingly as the complex formed by syphilitic serum and the lipoidal suspension which constitutes the "complement fixation antibody" in the Wassermann reaction. The fact that in this reaction the adsorbent precipitate is formed more rapidly in the ice chest than in the incubator is further evidence that the process is physical rather than chemical.

The Neisser-Wechsberg Phenomenon ("Complement Deviation")[70]

When in such a cytolytic system as is used in demonstrating complement fixation there is a considerable *excess of amboceptor* present, together with a limited amount of antigen and complement, there is commonly observed a failure of complement action; that is, no cytolysis takes place. Neisser and Wechsberg, who first described this phenomenon in 1901, attributed it to the binding of the complement by the excess amboceptors before it had an opportunity to unite with amboceptors already attached to the antigen. This explanation has not been acceptable because of the evidence furnished by the complement fixation reaction that complement does not unite readily if at all with amboceptors which have not already united with antigen.

With increasing appreciation of the relation of immunological reactions to colloidal chemistry processes the tendency has been to put the Neisser-Wechsberg phenomenon into the inhibition-zone reactions discussed in connection with agglutination and precipitation. In this case it is the excess antibody rather than the excess of antigen which inhibits the reaction. Gay has suggested that the precipitates which form in such serum-antigen mixtures may adsorb the complement and so "deviate" it. The more recent workers have made other suggestions. Thjøtta[71] believes that specific inhibiting antibodies arise during immunization which combine with dissolved antigen to form molecular complexes which have a marked tendency to adsorb complement. In other words, his specific inhibiting antibodies act like anticomplements. Pandit,[72] who reviews the earlier work, believes that the complement "deviation" does not depend on special inhibiting antibodies, but is the result of dissociation of the antigen-amboceptor complex which occurs when the amboceptor is in excess. He found that the complement is still free in a reaction mixture exhibiting the Neisser-Wechsberg phenomenon, showing that the "deviation" is not dependent on binding of complement by amboceptors as the foregoing authors believed. Hyde[72a] suggests that the excessive quantity of amboceptor supersensitizes the antigen so that the complement components are attracted to the cell out of the order that is necessary to effect lysis.

[70] This is an entirely distinct phenomenon from that of "complement fixation," but unfortunately numerous writers have caused confusion by incorrectly referring to the latter processes as complement deviation.

[71] *J. Immunol.*, **5**:1 (1920).

[72] *J. Hyg.*, **21**:406 (1923).

[72a] *Am. J. Hyg.*, **8**:730 (1928).

The Abderhalden Reaction [73]

This may properly be considered under the lytic reactions, for essentially it is a proteolytic reaction, indicated by the presence in the reacting fluid of evidences of protein disintegration. Furthermore, it would seem to be essentially an amboceptor-complement reaction, for it has been reported that sera giving positive reactions can be inactivated by heat and reactivated by normal serum,[74] although it is usually referred to as if depending upon the formation of specific ferments. However, Buadze [75] maintains that the Abderhalden reaction is not a complement fixation reaction, but an example of specific enzyme-formation.[76] In the case of digestion of tissue proteins, indeed, Abderhalden claims that the enzymes are derived from the same tissue as the one digested, for, he says, the serum of castrated animals will not form enzymes digesting testicle substance upon immunization with this material, nor will thyroidectomized animals produce enzymes digesting thyroid proteins.[77]

This reaction is based upon the hypothesis that the animal body reacts to the presence of foreign proteins by providing specific means of destroying them through proteolysis, and hence is fundamentally the same as the anaphylaxis reaction as conceived by Vaughan, Friedemann, Friedberger and others. It apparently differs from the other reactions of this class merely in that the methods used for determining the proteolysis are chemical rather than biological. The occurrence of a reaction is indicated by the production of diffusible products of protein hydrolysis, which may be detected by any one of several methods,[78] although most used is "ninhydrin" (triketo-hydrindene hydrate) [79] which reacts with any alpha-amino acid, the resulting condensation compound being a blue or violet color; or by observing the change in optical rotation that occurs in a solution of peptone under the hydrolytic action of the serum.[80]

The digestion seems to involve chiefly the proteins of the antiserum rather than the antigen, although under certain conditions there may

[73] For a general review *see* E. Abderhalden, "Schützfermente des tierischen Organismus." *Also,* review by J. Bronfenbrenner, in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, Chapter 79.

[74] *See* Stephan, *Münch. med. Wochschr.,* 61:801 (1914); Hauptman, *ibid.,* p. 1167; Bettencourt and Menezes, *Compt. rend. soc. biol.,* 77:162 (1916).

[75] *Fermentforschung,* 9:362 (1928).

[76] *See also* Abderhalden and Buadze, *Fermentforschung,* 10:111 (1928).

[77] Abderhalden and Wertheimer, *Fermentforschung,* 6:263 (1922).

[78] *See* Sellheim, *Klin. Wochschr.,* 4:247, 299 (1925).

[79] Chemistry of this reaction discussed by Retinger, *J. Am. Chem. Soc.,* 39:1059 (1917).

[80] A spectrophotometric method has also been recommended by Abderhalden [*Fermentforschung,* 9:214 (1927)]. The interferometer method of Paul Hirsch has been found unreliable by Haynes and Wolf [*J. Cancer Res.,* 11:301 (1927)].

be some digestion of the antigen.[81] Bronfenbrenner holds that the enzymes exhibit no selectivity, digesting both the antigen and the serum impartially.[82]

The mechanism of the reaction is not understood. Jobling and Petersen [83] have suggested that the antigen-antibody combination may adsorb or bind the antiproteases of the serum, so that the normal protease digests the serum proteins. Or it may be that union of antigen and antibody activates the complement, or binds it to the antibody so that it digests either the antibody or other proteins of the serum. It also is suggested that enzymes are set free from the tissues injured by the specific protein, or by disease, and digest the foreign protein or the cellular proteins that may have escaped from the tissues into the blood stream.

Serum treated with various inert, finely divided particles, such as kaolin, starch, silicates, etc., may acquire the property of giving positive reactions. This is another point of resemblance to anaphylatoxin formation, and against the specificity of the reaction, indicating that the antigen merely acts as a non-specific adsorbent.

Although it has been claimed by Abderhalden and his followers that this is a most delicate and specific reaction, most of the investigators who have tried to use it have failed to establish this specificity.[84] By far the most satisfactory results have been recorded in the diagnosis of pregnancy by means of placental antigen. This may be explained by the fact that the protease activity of the serum seems to be increased in pregnancy,[85] and hence the reaction with placenta is more marked than with the serum of non-pregnant individuals. But simply shaking normal serum with kaolin or other foreign substances may cause it to give strong reactions with placenta antigen (Wallis).[86]

Careful quantitative studies of the setting free of amino acids by serum incubated with placenta, by Van Slyke and his associates, also showed a lack of demonstrable specific proteolysis by pregnancy serum.[87, 88]

[81] Abderhalden, *Fermentforschung*, **6**:230 (1922).

[82] Supported by Smith and Cook, *J. Infectious Diseases*, **18**:14 (1916). De Waele states that it is the serum globulin that is digested [*Compt. rend. soc. biol.*, **76**:627 (1914)].

[83] *J. Lab. Clin. Med.*, 1:172 (1915).

[84] Richard Stephan and Erna Wohl, *Z. exp. Med.*, **24**:391 (1921). Tanaka, *Gann*, 17:7 (1923). Berthold Oppler, *Biochem. Z.*, 75:211 (1916). Hogler and Serio, *Wien. Arch. inn. Med.*, **7**:571 (1924). Wollman *et al.*, *Ann. inst. Pasteur*, **38**:114 (1924); Kleesattel, *Klin. Wochschr.*, **5**:796 (1926).

[85] Sloan, *Am. J. Physiol.*, **39**:9 (1915).

[86] *See* Wallis, *Quart. J. Med.*, **9**:138 (1916); Bronfenbrenner, *J. Lab. Clin. Med.*, 1:79 (1915); 1:573 (1916).

[87] Hulton, *J. Biol. Chem.*, **25**:163 (1916).

[88] Van Slyke, *Arch. Int. Med.*, **19**:56 (1917); *J. Biol. Chem.*, **23**:377 (1915). *See also* Kupelweiser *et al.*, *Biochem. Z.*, **178**: 298 (1926); and Singer, *ibid.*, p. 332.

O. J. Elsesser,[89] working in my laboratory with the purified vegetable proteins of Osborne, found that at best the specificity of the reaction was less than that of the anaphylaxis reaction, and there were many absolutely non-specific and irrational reactions. As these pure proteins furnish a much more appropriate material for studying specificity than the tissues or sera commonly used, it would seem that the results thus obtained are excellent proof of the uncertainty and unreliability of the reaction.

The Meiostagmin Reaction [90]

This was devised by Ascoli and Izar, and is based on the principle that the reaction of antigens with their specific antibodies results in lowering the surface tension of the solution in which this reaction occurs, which may be demonstrated by counting the number of drops of the fluid per minute, under constant conditions. These authors used the stalagmometer to measure the change in surface tension, and arbitrarily decided that an increase of two drops per minute constitutes a positive reaction after two hours' incubation of the reacting mixture; the increase is seldom above eight drops. The antigens used are soluble in alcohol but their nature is unknown; the antibody involved in the reaction is referred to as the meiostagmin, but its relation to other antibodies is likewise unknown. There has been an attempt to explain the reported observations as the effect of the action of lipoids, linoleic-ricinoleic-acid mixtures having been used as antigen by some experimenters.[91]

Gouwens [90] found the Du Nouy surface tension apparatus preferable to the stalagmometer for such work, but was unable to demonstrate by the meiostagmin reaction the presence of specific antibodies in *B. paratyphosus B* immune rabbit serum of known high antibody content. Other workers have also failed to corroborate the original claims for this reaction,[92] and it now seems probable that the fundamental principle of a measurable and constant change in surface tension occurring when antibodies react with specific antigens, is not well founded. Loeb [91] believes that the meiostagmin reaction merely indicates that the serum in some pathological states has less capacity than normal serum to counteract or inhibit the lowering of surface tension by the lipoids used as "antigens." This is therefore not an immunity reaction, but merely a non-specific phenomenon of colloidal chemistry, dependent on unknown changes in the serum in disease.

The Epiphanin Reaction

Weichardt [93] reported that there is an acceleration in the rate of diffusion in a solution in which antigen and antibody react, and stated that this change could be detected by observing a shift towards the acid side of a previously neutral mix-

[89] *J. Infectious Diseases*, 19:655 (1916).
[90] Full review of literature and bibliography given by Gouwens, *J. Infectious Diseases*, 31:237 (1922).
[91] N. Blumenthal, *Z. Immunität.*, 24:42 (1915); L. F. Loeb, *Biochem. Z.*, 136:190 (1923).
[92] Maiweg and Eichholtz, *Biochem. Z.*, 140:555 (1923).
[93] *Z. Immunitäts.*, 4:644 (1910); *Berl. klin. Wochschr.*, 48:1935 (1911).

ture of sulfuric acid and barium hydroxid. Phenolphthalein in combination with a catalytic agent, strontium chlorid, is used as the indicator. Colloidal substances are said to alter the surface tension of the suspension of finely divided barium sulfate particles so as to increase the adsorption of H ions. In this way the point of neutralization is shifted accordingly as the amount of adsorption is large or small. Burmeister,[94] Reich[95] and others[92] have been unable to corroborate the applicability of such a reaction, and of recent years nothing has been published concerning it. Nevertheless, some observers have been able to corroborate the fundamental principle (Kraus, Amiradzibi) of a change of rate of diffusion in solutions in which antibodies react with antigens.

Recapitulation

The dissolution of bacteria and other cells which may be accomplished by the serum of animals immunized to these cells, depends on the combined action of two agents present in such an immune serum. One, the immune body, unites with the antigenic cell, and this combination in turn unites with a second, complementary agent, which accomplishes the disintegration of the cell. These two agents are known respectively as (a) the immune body (or amboceptor or sensitizer or sensitizin); and (b) the complement or alexin. The immune body which sensitizes the antigen in this reaction (i.e., the amboceptor or sensitizin) is perhaps identical with the immune body responsible for the precipitin and agglutinin reactions. It is formed in response to the introduction of the antigen into the body and reacts specifically with that antigen, and exhibits the properties common to immune antibodies in general, including the consideration that it is quite possible that "immune bodies" do not exist as definite substances, but merely represent the effects of a certain colloidal state of the serum proteins.

The complement (or alexin) which has the lytic effect is present in normal serum, is not increased in amount during immunization, and is not specific, reacting with any antigen-amboceptor compound to which it is exposed. It is a relatively large colloidal complex, and apparently consists of at least two protein components, one, the "mid-piece," being a globulin, the other, the "end-piece," is an albumin, and possibly lipoids enter into the complex. Other components may also participate in constituting the complement, and some of the properties of complement may be exhibited by artificial colloidal mixtures of lipoids and salts. Perhaps because of its complex structure, complement is highly susceptible to inactivation by heat and other physical and chemical agents, and in many respects resembles enzymes, except in that it par-

[94] *J. Infectious Diseases*, 12:459 (1913).
[95] *Z. Immunitäts.*, 18:480 (1913).

ticipates in reactions according to the law of definite proportions. However, the destruction of cells accomplished by complement-amboceptor reaction has not been shown to depend on proteolysis of these cells.

The destruction of cells by amboceptor-complement action is but a single feature of a more general reaction, for any antigenic protein will unite with the specific amboceptor, and the resulting antigen-antibody complex will unite with complement quite as well as if the antigen were a cell. Probably in this case, when the antigen is soluble, some degree of proteolysis is accomplished by the complement, just as cytolysis occurs when the antigen is in cellular form. This reaction is extremely delicate and specific, wherefore it has been found of much value in immunological investigations and in clinical medicine in the form of the Bordet-Gengou reaction of "complement fixation" and the reaction derived therefrom by Wassermann for diagnosis of syphilis.

This complement-fixation reaction seems to be a typical colloid-chemical reaction, dependent upon the physical state of the colloids and the pH and electrolyte content of the fluid in which the colloids are dispersed. The complement function is manifested only when the colloidal particles resulting from union of antigen and antibody are of a certain size and when electrolytes are present, and in some reactions artificially constructed colloidal emulsions may be made to serve either as antigen or as complement. Furthermore, the fact that the adsorbent precipitate responsible for the complement fixation reaction takes place more rapidly in the ice chest than in the incubator is in favor of its being physical rather than chemical in the usual sense.

The so-called Abderhalden reaction is evidence that proteolysis is accomplished when antisera and antigen react under suitable conditions, although most of the proteolysis seems to occur in the proteins of the immune serum rather than in the antigenic proteins. The amount of proteolysis is slight, and it is not yet agreed whether the digestion depends on the action of the same complement that accomplishes other lytic reactions, or on special "defensive ferments." Under ordinary conditions, at least, this reaction lacks reliable specificity.

CHAPTER VIII

THE WASSERMANN REACTION[1] AND RELATED REACTIONS WITH SYPHILITIC BLOOD

This reaction is commonly referred to as the complement fixation reaction for syphilis because it is based on the Bordet-Gengou reaction, and when first described was supposed to be a true complement fixation reaction depending on the reaction of syphilitic virus as antigen with antibodies specific for this virus. Wassermann devised the reaction before the cultivation of the specific organism, the *Treponema pallidum* (or *Spirochæta pallida*), and to secure the antigen took advantage of the fact that the livers of fetuses with congenital syphilis are swarming with these organisms. Extracts of the livers of such fetuses were therefore used for the antigen, and were found to give specific complement fixation reactions when the serum of syphilitics was used to furnish the antibody, no complement fixation being ordinarily obtained with normal serum or with the serum of persons with other diseases. The success of these results at first seemed to prove that this reaction must be a true complement fixation reaction, in which the union of the specific syphilitic antigen with an antibody for syphilitic virus produces a complement-fixing sensitized antigen, as in the typical Bordet-Gengou reaction.

Further investigation, however, showed that the Wassermann reaction is not at all the same thing as the Bordet-Gengou reaction of complement fixation. It was soon found by Landsteiner and others that extracts of normal livers give just as good, specific reactions as extracts of syphilitic livers, and then it was learned that alcoholic extracts of almost any organ will take the place of the syphilitic antigen, extracts from beef hearts being especially effective. Moreover, when Noguchi succeeded in devising means for cultivating the *Treponema,* it was found that aqueous extracts of these organisms were entirely ineffective when used as antigen with the serum of human syphilitics as a source of antibody, although such serum gave strongly positive Wassermann reactions.[2] These and many other observations have

[1] The early literature is fully reviewed by Noguchi, "Serum Diagnosis of Syphilis and Luetin Reaction," Philadelphia, 1912. Lippincott.

[2] F. Klopstock [*Deut. med. Wochschr.*, **52**:226 (1926)] has been more successful with antigens made by alcohol extraction of *Trepomema,* but extracts from *Spirocheta dentium* were equally effective.

seemed to indicate that the Wassermann reaction, although almost specific evidence of syphilitic infection, is not a true complement-fixation reaction demonstrating the presence of syphilitic antibodies in the serum of the person infected with syphilis. The actual nature of the reaction is still obscure, but the present tendency is to consider it dependent upon physico-chemical and colloidal phenomena.[3]

The Nature of the "Antigen"

Extended investigation of these non-specific antigens which give specific complement fixation with syphilitic sera, has shown them to be related to the lipoids, especially to the lecithin, as indicated by the fact that one of the most efficient "antigens" is the acetone-insoluble fraction of the tissue lipoids. Klein and Fraenkel[4] believe the "antigen" of ox heart extracts to be a combination of lecithin with cholesterol and small amounts of a soap-like substance similar to jecorin. An acetone-precipitated "antigen" of this class is not a true antigen in the sense of possessing the capacity to incite formation of antibodies, however, for fixation antibodies are not developed in animals injected with such a lipoid mixture, which has been shown to be entirely efficient in the Wassermann reaction.[5]

The antigenic value of the alcohol-soluble fraction of different liver extracts varies nearly directly with its power to combine with iodin, according to Noguchi and Bronfenbrenner. This would indicate that the unsaturated fatty acids are important in the reaction, but their observation was not corroborated by Browning,[6] and saturation of the

$$\begin{matrix} & & H & H \\ & & | & | \\ -C & = & C & - \end{matrix}$$

lipoids with hydrogen, whereby the double bond —C=C— is reduced

$$\begin{matrix} & & H & H \\ & & | & | \\ -C & - & C & - \end{matrix}$$

to the single bond form —C—C— does not remove the activity of the

$$\begin{matrix} & & H & H \\ & & | & | \\ -C & - & C & - \end{matrix}$$

lipoids, indicating that this does not depend on the unsaturated carbon atoms.[7] Such hydrogenated lipoids are, however, more "anticomplementary" than untreated lipoids.

Crude lecithins from different sources vary in efficiency, heart lecithin being more active than liver lecithin, brain and egg yolk lecithin following. Pure lecithin is not effective, the activity of lipoid solutions

[3] *See* review of physico-chemical theories by Epstein and Paul, *Kolloid Z.*, 29:310 (1921); *Arch. Hyg.*, 90:98 (1921).
[4] *Münch. med. Wochschr.*, 61:651 (1914).
[5] Fitzgerald and Leathes, *Univ. Calif. Pub. Path.*, 2:39 (1912).
[6] Browning *et al.*, *Z. Immunitäts.*, 14:284 (1912); *J. Path. Bact.*, 16:135 and 225 (1911).
[7] C. L. A. Schmidt and S. E. Coffey, *J. Infectious Diseases*, 32:119 (1923).

apparently depending upon some other substance which is difficult to separate from lecithin (MacLean).[8] In favor of the view that it is the lecithin itself that is responsible, however, is the observation of Nathan that the activity of the antigen is reduced by exposure to cobra venom, which has an enzyme that splits lecithin.[9] Even vegetable lipoids extracted from pea flour may serve as an efficient "antigen," [10] and the lipoids of *Spirocheta dentium* and *Treponema pallidum* are equally good antigens.[11] Addition of cholesterol to the lipoid solutions increases greatly their activity [6] although cholesterol itself is practically without antigenic activity, a fact which suggests that the physical properties of the colloidal lipoidal emulsions used as antigen are of first importance in determining their activity, for cholesterol greatly modifies the physical properties of the lipoidal mixture. The amount of cholesterol necessary is, however, very small, varying from 0.014 to 0.033 per cent according to Frank [12] and addition of cholesterol to normal serum will not make it give positive reactions.

Having observed a certain degree of specificity with antisera for lipoids from different organs, Steinfeld compared heart and brain lipoids as Wassermann antigens, and found that the brain lipoids often gave positive reactions with the spinal fluid in cerebrospinal syphilis, although not with the serum of the same patients, supporting the view that the Wassermann reaction depends on autoantibody formation against tissue lipoids.[13]

The antigen is employed as a colloidal suspension in salt solution, and its efficiency depends greatly upon the size of the particles in the suspension,[14] indicating that the function of the antigen depends upon the surface development of the suspension colloid.[15] The efficient lipoid antigen seems to consist of a mixture of particles varying in size from coarse suspensions to true colloidal dispersion, with a relatively large proportion on the border-line between the two stages,[16] which makes for the lack of stability characteristic of and apparently essential for such "antigens" (Epstein and Paul).[17]

[8] Monographs on Biochemistry, "Lecithin and Allied Substances," London, 1918. Longmans, Green and Co.
[9] Nathan, *Z. Immunitäts.*, **26**:154, 582 (1917); **35**: 392 (1923).
[10] R. M. Walker, *J. Path. Bact.*, **21**:184 (1917).
[11] F. Klopstock, *Deut. med. Wochschr.*, **52**:226 (1926).
[12] *Klin Wochschr.*, **1**:419 (1922); *Wien. Arch. inn. Med.*, **10**:357 (1925).
[13] Steinfeld, *Verhandl. 38 Kongress deut. Gesell. inn. Med.*, **1926**, p. 72.
[14] See Freiwirth, *Z. Immunitäts.*, **46**:157 (1926).
[15] For two antigens of the same turbidity, the activity varies inversely as the pH [Douris, Mondain and Beck, *Compt. rend. soc. biol.*, **98**:507 (1928)].
[16] This fact may explain the reported successful substitution for the Wassermann antigen of various colloidal suspensions of soaps and salts, or even of phenol [Ninni and Molinari, *Centr. Bakt.*, **104**:502 (1927); *Pathologica*, **20**: No. 442 (1928)].
[17] *Kolloid Z.*, **29**:310 (1921); *Arch. Hyg.*, **90**:98 (1921).

The Nature of the Reacting Agent ("Amboceptor") of the Serum

As for the substance in the syphilitic serum which participates in the Wassermann reaction, it would seem to be related to the globulins, which are decidedly increased in the blood and spinal fluid of syphilitics,[18] especially the euglobulin.[19] If the globulin and albumin of a positive serum are separated, the isolated globulin contains all the reactive agent, the albumin giving negative reactions (Kapsenberg).[20] Oeller and Schierge [21] could not isolate a single protein fraction exhibiting all the activity. Most of it is thrown out of solution when serum is treated with CO_2, which precipitates euglobulin with some pseudoglobulin (Mackie).[22] According to Stern [23] only a part of the euglobulin fraction is active. During antisyphilitic treatment there is a fall in the globulin content of the blood associated with the disappearance of the Wassermann reaction.[24] Kafka [25] reports that spinal fluid may give a positive Wassermann reaction when it contains no appreciable amount of globulin, and this is confirmed by Forssman,[26] who therefore questions the dependence of the reaction upon the globulins. However, it is well established that a characteristic feature of the spinal fluid in syphilis is an increase of globulin, identical with that of the serum as shown by its immunological behavior (Neymann and Hektoen),[27] and Herrold [28] found the active agent in syphilitic spinal fluids in the euglobulin fraction. Ellinger [29] even found that immunization with syphilitic spinal fluid engendered antibodies reacting with Wassermann-positive spinal fluids. In any case, the active agent does not pass through a collodion membrane, even under pressure,[30] and hence is certainly associated with the colloids.

Most authors follow the lead given by Friedemann,[31] who found the globulin, or rather, a globulin-soap compound, responsible for positive Wassermann reactions, which the serum albumin tends to prevent. Forssman's [32] study of the chemical nature of the active agent in the

[18] *See* Rowe, *Arch. Int. Med.*, **18**:455 (1916); Schiff and Roser, *Monatschr. Kinderheilk.*, **19**:15 (1920).
[19] Harold, *J. Army Med. Corps.*, **39**:83 (1922); **40**:44 (1923); Ruppel, *Deut. med. Wochschr.*, **49**:46 (1923).
[20] *Ann. inst. Pasteur*, **35**:648 (1921); *Z. Immunitäts.*, **39**:3 (1924); **42**:284 (1925).
[21] *Z. Immunitäts.*, **36**:59 (1923); **40**:393 (1924).
[22] *J. Hyg.*, **21**:386 (1923).
[23] *Biochem. Z.*, **144**:115 (1924).
[24] Bircher and McFarland, *Arch. Dermatol. Syphilis*, **5**:215 (1922).
[25] *Z. Immunitäts.*, **37**:315 (1923).
[26] *Biochem. Z.*, **177**:243 (1926); *Acta Path. Microbiol. Scand.*, **4**:198 (1927).
[27] *J. Nervous Mental Diseases*, **56**:16 (1922).
[28] *J. Am. Med. Assoc.*, **81**:203 (1923).
[29] *Z. physiol. Chem.*, **132**:134 (1924).
[30] Trenti, *Policlinico*, **32**:867 (1925); *Centr. Bakt.*, **97**:57 (1925).
[31] *Z. Hyg.*, **67**:279 (1910).
[32] *Biochem. Z.*, **121**:180 (1921).

serum also led him to the conclusion that, although it is precipitated with the globulins, it is not a globulin, but more probably a lipoid associated with the globulin, and Hartley [33] found that ether extraction of syphilitic serum makes it Wassermann negative. This could not be confirmed by Reiner and Török,[34] who located the active agent in the pseudoglobulin. Weston [35] found that the active substance of syphilitic spinal fluid is not identical with the globulin which is responsible for the colloidal gold reaction of such fluids.

Schmidt [36] ascribes the reaction to the physico-chemical properties of the globulins of the syphilitic serum, which, he believes, possess a greater affinity for the colloids of the antigen than normal globulins; this affinity is held in check in normal serum by the albumins of the serum, which in syphilis are relatively or absolutely decreased. That physico-chemical factors do play a part is evidenced by the common observation that the turbidity of the antigen suspension is closely related to its efficiency, clear solutions being less active. Slight changes in H-ion concentration will change a reaction from negative to positive, or the reverse; and neutral salts can change a negative to a positive reaction, but not the reverse (Cumming).[37] Even the mere treatment of a negative serum with ether may cause it to give positive reactions (Forssman),[38] possibly by causing a change in the colloidal state of the lipoproteins of the serum. Unlike the "antigen," the "amboceptor" is not flocculated by electrolytes in small concentrations, and hence it is apparently an emulsoid or hydrophilic colloid, whereas the "antigen" behaves like a suspensoid colloid in which the disperse phase is solid. Stern [39] could find no difference in respect to the electrical charge of the active globulin fraction of positive sera and normal globulin. As treatment of negative serum with tannin renders it positive, it is possible that activity may be dependent on dehydration processes.

The lipoids in syphilitic sera are said by Peritz [40] to be increased, but the lipoid content and the antibody titer do not show any constant relation (Bauer and Skutezky).[41] The cholesterol content of syphilitic blood shows no evidence of a quantitative relation to the Wassermann reaction,[42] although generally increased, whereas the lipolytic activity

[33] *Brit. J. Exp. Path.*, 6:180 (1925).
[34] *Z. Immunitäts.*, 53:532 (1927).
[35] *Am. J. Insanity*, 76:393 (1920).
[36] *Z. Hyg.*, 69:513 (1911). *See also* Hirschfeld and Klinger, *Z. Immunitäts.*, 21:40 (1914).
[37] *J. Infectious Diseases*, 18:151 (1916); *see also* McManus, *J. Immunol.*, 8:443 (1923).
[38] *Biochem. Z.* 121:180 (1921).
[39] *Biochem. Z.*, 144:115 (1924).
[40] *Z. exp. Path.*, 8:255 (1910).
[41] *Wien. klin. Wochschr.*, 26:830 (1913).
[42] Weston, *J. Med. Res.*, 30:377 (1914); Stein, *Z. exp. Med.*, 3:309 (1914).

is decreased.[43] According to Frankenthal,[44] in Wassermann positive sera the lipoids are chiefly in the albumin fraction; in normal sera they are in the globulin fraction.

McIntosh [45] says that the active component differs from typical antibodies in not passing through collodion or porcelain filters, and there are many who hold that the reacting substance is a product of tissue disintegration. For example, Maho found that minute quantities of glycine, leucine and tyrosine added to normal serum cause it to give a positive reaction. This has been confirmed by Bachmann,[46] who also found that the ninhydrin test for amino acids runs parallel with the strength of the Wassermann reaction in syphilitic sera. Hans Much [47] has also observed that intravenous injection into rabbits of leucine, $HgCl_2$ or tubercle bacillus lipoids, causes the blood to become temporarily Wassermann positive, but as a matter of fact the blood of normal rabbits not infrequently gives positive Wassermann reactions.[48] Reinvestigating the preceding observations Fowweather and Gordon [49] could not make rabbits' serum Wassermann positive by injecting amino acids, and they found no relation between amino acid content and Wassermann reaction in human serum. Solari [50] found the colloidal and non-colloidal nitrogen the same in syphilitic and normal serums. Forssman [51] reports that formalin inactivates the Wassermann reactive agent irreversibly, which indicates that it does not depend on either free amino acids or terminal amino acids in protein molecules.

That true antibodies are concerned in the Wassermann reaction has been doubted ever since the non-specific character of the antigen was first pointed out. In favor of their participation is the fact that the serum of rabbits immunized with congenital syphilis livers contains an antibody giving the Wassermann reaction, exactly like the serum of syphilitics.[52] On the other hand, the actual substance of pure cultures of spirochetes does not ordinarily act as antigen with syphilitic sera in the Wassermann reaction (Noguchi). It is possible that when syphilitic liver extracts are used as antigen in the Wassermann reaction, we have a true Bordet-Gengou reaction of complement fixation with the syphilitic substance present in this extract, in addition to the usual reaction

[43] Weiss and Dorle, *Biochem. Z.*, 171:225 (1926).
[44] *Z. Immunitäts.*, 42:501 (1925).
[45] *Z. Immunitäts.*, 5:76 (1910).
[46] *Z. Immunitäts.*, 33:233 (1921).
[47] Much and Schmidt, *Deut. med. Wochschr.*, 47:552 (1921).
[48] Browning, *Brit. Med. J.*, 1:239 (1915).
[49] *Brit. J. Exp. Path.*, 8:93 (1927).
[50] *Anales assocn. quim. Argentina*, 12:421 (1924).
[51] *Acta Path. Microbiol. Scand.*, 1:5 (1924).
[52] Citron and Munk, *Deut. med. Wochschr.*, 36:1560 (1910); Eiken, *Z. Immunitäts.*, 24:188 (1915); Meyer, *Z. Immunitäts.*, 31:278 (1921).

which is accomplished by the use of lipoids as antigen and which alone is concerned in the usual technic with non-syphilitic lipoidal antigens.

The Wassermann Reaction as an Autoimmunization

A new phase of the study of the Wassermann reaction was initiated by Taniguchi's demonstration that the "Forssman antibody," generated on immunization with "heterophile antigens" gives Wassermann reactions, and Landsteiner's discovery that crude tissue lipoids admixt with a foreign protein constitute antigens which engender antibodies that react with these lipoids.[53] Sachs [54] and his associates applied this fact to the Wassermann reaction, because this also is a reaction in which tissue lipoids function like an antigen in reacting with an immune serum. They found that rabbits injected with crude lipoids mixed with swine serum develop a change in their serum so that it will give positive Wassermann reactions and the flocculation reactions of Sachs-Georgi and of Meinicke, quite like the serum of a man with syphilis. They therefore venture the hypothesis that, after all, these reactions given by the serum of syphilitics really are immunological reactions against lipoid-protein antigens, a process of autoimmunization as suggested long ago by Weil and Braun.

These antigens may conceivably be constituted by the lipoid-protein of the spirochetes, or by tissue lipoids combined with spirochete proteins, or spirochete lipoids with tissue proteins,[55] or even the lipoids and proteins of the tissues disorganized by the disease.[56] Despite numerous studies and discussions from Sach's laboratory,[57] and others, it has not yet been decided which of these is the agent or antigen which leads to the characteristic changes in the blood in syphilis.

F. Klopstock has demonstrated that alcoholic extracts of spirochetes will engender antibodies reacting with these extracts, and also with alcoholic extracts of tissues, but Sachs and Weil have obtained Wassermann reactions with the serum of rabbits immunized with alcoholic extracts of normal organs mixed with swine serum, using these same extracts as antigens in the tests. Even immunizing with killed trypanosomes will produce sera giving positive Wassermann and flocculation reactions (Landsteiner and van der Scheer),[58] possibly because these killed trypanosomes contain lipoids and foreign proteins in proper con-

[53] Discussed in Chapters II and III.
[54] Sachs, Klopstock and Weil, *Deut. med. Wochschr.*, **51**:589, 1017 (1925); *see also* Sachs, *Klin. Wochschr.*, **4**:1630 (1925).
[55] *See* Heimann, *Arch. exp. path. Pharmakol.*, **115**:64 (1926).
[56] *See also* Hadjopoulos and Burbank, *J. Lab. Clin. Med.*, **12**:973 (1927).
[57] *See* review by Sachs, *Deut. med. Wochschr.*, **53**:394 (1927).
[58] *J. Exp. Med.*, **45**:465 (1927).

ditions. Apparently the great variety of tissues and cells which can furnish extracts that will function in the Wassermann reaction depends on the lack of specificity of the lipoids.

It cannot yet be stated positively that the Wassermann reaction with the serum of human syphilitics is truly an antibody reaction to lipoid protein complexes, despite the similarity to the results obtained with the serum of rabbits immunized with lipoids and proteins. Rabbits sometimes give the same sort of Wassermann reactions without previous immunization, and they apparently have an especially labile lipoid metabolism that makes them peculiarly adapted to produce the necessary conditions in their serum to give the Wassermann reaction. Mackie and Watson [59] find that this is true for many other mammals, whereas man, rat, and guinea pig differ in not giving spontaneous Wassermann reactions.[60] In accordance with this is the fact that Martin [61] and Förtig [62] were unable to produce a positive Wassermann reaction by immunizing men with lipoids and foreign serum, that Heronimus and Awrech [63] failed with rats, and that Frei and Henning were unsuccessful with guinea pigs.[64]

The Nature of the Reaction

Whether the complement is destroyed by enzymes,[65] or is inhibited by anti-complement present in syphilitic serum, or is destroyed by some toxic substance in the serum,[66] or is physically bound by the reaction product formed by the antigen and the serum amboceptor, are matters still under discussion. A favorite interpretation of the Wassermann reaction, which seems to harmonize with the known facts, is that there is a precipitation of serum globulin by the lipoidal colloids of the antigen, and adsorption of the complement by this precipitate. In favor of this hypothesis is the fact that examination of Wassermann reaction mixtures of syphilitic serum and antigen by the ultramicroscopic method shows the presence of precipitates even when these precipitates are not macroscopically visible (Jacobstahl).[67]

Apparently the globulins of the serum in syphilis are altered in some

[59] *J. Hyg.*, **25**:176 (1926).
[60] Even in normal human serum, however, a small amount of activity can be demonstrated in the proteins precipitated by CO_2, which is the basis for the hypothesis of Mackie and Watson [59] that the Wassermann reaction is due to an increase of a "lipoidophile" antibody naturally present in the serum in a masked state and in minimal amounts.
[61] *Dermatol. Z.*, **46**:176 (1926).
[62] *Z. Immunitäts.*, **52**:328 (1927).
[63] *Z. Immunitäts.*, **53**:541 (1927).
[64] *Klin. Wochschr.*, **6**:1906 (1927); *Z. Immunitäts.*, **55**:19 (1928).
[65] Manwaring, *Z. Immunitäts.*, **3**:309 (1909).
[66] Kiss, *Z. Immunitäts.*, **4**:703 (1910); Thiele and Embleton, *Z. Immunitäts.*, **16**:430 (1913).
[67] *Münch. med. Wochschr.*, **57**:689 (1910).

specific but as yet unknown way, whereby they acquire in greatly increased degree the capacity to form this adsorbent precipitate,[68, 69] or lose their capacity to act as protective colloids and prevent that aggregation of particles which favors the adsorption of complement.[70] Alterations in the lipoids also seem to play a part, for it is known that conditions that modify the serum lipoids also modify the reaction. There seems little doubt that the reaction is not chemical but physical, and the union of complement to antibody follows essentially the laws of adsorption (Walker).[68] As with the other colloidal reactions of this class, the presence of electrolytes is an essential feature (Holker).[70] If the reaction is carried out at a pH more alkaline than 7.6 there will be reduction in the amount of complement fixation.[71]

The significance of both the lipoids and the proteins is generally accepted. This is emphasized by Zinsser in the following statement: "In the Wassermann reaction, as we know, complement is fixed by a combination of syphilitic serum and various lipoidal suspensions, which may be entirely non-specific in origin. When the reaction occurs, as Jacobstahl and, later, Bruck have shown, a precipitation occurs which can be seen in the ultramicroscope. Furthermore, this precipitation takes place more rapidly in the ice-chest than in the incubator, which strikingly suggests that the reaction is an adsorption rather than a true chemical union. It is this precipitate which fixes the complement; whether or not this is due to quantitatively increased globulin or to purely physical change in the syphilitic serum, is a matter which we cannot discuss at present. Whatever it is, it is unquestionable that the availability of the antigen for the Wassermann reaction depends not only upon its lipoidal nature but also on its state of dispersion. Since it is not possible, as we have found, to make available antigens for this reaction with non-lipoidal substances, like mastic, gelatin, gum arabic, silicic acid, albumins, and a number of other substances, even when in dispersion more or less similar to that of the Wassermann antigen, it seems that the secret of the Wassermann antigen must lie in the fact that substances of the chemical and physical constitution of lipoids when brought into a definite state of dispersion offer surface tension conditions not easily obtained with colloids of another nature. It is, therefore, at least in our opinion at present, the physical condition of the Wassermann antigen which makes it available for the test, a physical

[68] Walker, *J. Path. Bact.*, 21:184 (1917).
[69] *See* Nathan, *Z. Immunitäts.*, 27:219 (1918).
[70] Holker, *J. Path. Bact.*, 25:281 (1922).
[71] Hill, McKinstry, and Parker, *J. Path. Bact.*, 28:47 (1925).

condition which is secondarily dependent upon the chemical nature of the dispersed substance. The importance of the state of dispersion and therefore the surface tension properties is quite apparent from the fact noted by many Wassermann workers that a considerable difference in the fixing power of the antigen may be obtained by, in one case, adding the salt solution to the alcoholic extract quickly, and in another case adding it very slowly, the two separate preparations showing, one a very transparent, the other a very turbid, condition." Zinsser concludes his discussion of the Wassermann reaction with the following summary: "While, therefore, we are still considerably in the dark concerning the true nature of the Wassermann reaction, we may state with safety that there is present in the syphilitic serum a substance which leads to the formation of a precipitate when brought into contact with properly prepared alcoholic extracts of normal tissues. The so-called antigenic substances, then, are probably lipoidal in nature, or, at any rate, represent lipoid-protein complexes. The precipitate formed, probably because of its physical properties, is now capable of fixing alexin."

Chemical Changes in the Blood in Syphilis

The changes in character of the blood serum in syphilis are sufficient to give not only immunological but also frank chemical or physicochemical manifestations. For example, Bruck [72] states that the precipitate obtained when nitric acid is added to syphilitic serum is more abundant and of a characteristic gelatinous appearance. Platinum chloride also produces a heavier precipitate in syphilitic sera (Brown and Iyengar).[73] The globulin responsible for the Wassermann reaction is said to be precipitated more readily by ammonium sulfate and other reagents.[74] The viscosity of syphilitic serum is increased because of the increase in euglobulin (Holker).[75] According to von Dungern [76] the heat coagulation of syphilitic serum is prevented by a relatively smaller quantity of an alkaline solution of indigo than is the case with normal serum.[77] Syphilitic serum also flocculates on addition of appropriate colloidal suspensions which will not coagulate normal serum (Vernes).[78]

[72] *Münch. med. Wochschr.*, **64**:25 (1917).
[73] *Indian J. Med. Res.*, **3**:95 (1915).
[74] Heller, *Biochem. Z.*, **90**:166 (1918); McDonagh, *Proc. Royal Soc. Med.*, **9**: pt. 1, 191 (Derm. Sect.) (1916).
[75] *J. Path. Bact.*, **24**:413 (1921).
[76] *Münch. med. Wochschr.*, **62**:1212 (1915).
[77] This statement disputed by Flood [*J. Immunol.*, **2**:69 (1916)]; and Fujimoto [*J. Immunol.*, **3**:11 (1918)].
[78] *Compt. rend. acad. sci.*, **167**:383 (1918).

Landau [79] states that syphilitic serum has a heightened power to decolorize and clear up an iodin precipitate produced in the serum.[80]

Flocculation Reactions [81]

On the basis of these changed properties of the serum in syphilis, numerous more or less specific reactions have been devised,[82] and although these are for the most part inferior in clinical diagnostic value to the refined Wassermann reactions, yet they are of interest here as showing that there must be considerable changes in the colloids of the blood serum in syphilis. Among them may be mentioned briefly the following:

Klausner's Serum Reaction.—When distilled water is added in certain proportions to fresh serum, a distinct flocculent precipitate separates out in a few hours, and this property is much more marked in syphilitic than in normal sera. While not specific for syphilis, this reaction is almost invariably present in certain stages of syphilis. This property is not due to the excess of globulin present in syphilitic sera, according to the later studies of Klausner,[83] who believes that the high lipoid content of syphilitic serum is responsible.

Porges-Hermann-Perutz Reaction.—If equal parts of a 2% solution of sodium glycocholate and an alcoholic cholesterol suspension (0.4%) are added to inactivated serum from syphilitic patients, a coarse precipitate forms, while with normal serum there occurs little or no precipitate.[84] Little is known concerning the nature of this reaction.

Coagulation Reaction.—This was described by Hirschfeld and Klinger,[85] and depends on the fact that tissue extracts digested with syphilitic serum lose their ability to coagulate blood. The effect is believed to depend on adsorption of the coagulating lipoids of the tissue extract by serum constituents, and hence is fundamentally similar to the Wassermann reaction.

Meinicke Reactions.[86]—These are based on the hypothesis that the colloids of alcoholic extract of tissues disturb the isotonicity of saline solutions, permitting the union of serum globulins and lipoids, the reaction being greater with syphilitic than with non-syphilitic sera. If normal and syphilitic sera are precipitated, respectively, with dilute colloidal solutions of a lipoidal antigen in distilled water, the precipitate which forms in the globulin-rich syphilitic serum is less soluble in salt solution than that from the normal serum.[87]

[79] *Wien. klin. Wochschr.*, **26**:1702 (1913).
[80] Not corroborated by Stillians and Kolmer [*J. Am. Med. Assoc.*, **64**:1964 and 1966 (1915)]
[81] For general review *see* A. Klopstock, *Ergeb. inn. Med. Kinderheil.*, **28**:211 (1925).
[82] *See* review by Levinson, *Am. J. Syphilis*, **5**:414 (1921).
[83] *Biochem. Z.*, **47**:36 (1912).
[84] *See* Gammeltoft, *Deut. med. Wochschr.*, **38**:1934 (1912); Ellermann, *Deut. med. Wochschr.*, **39**:219 (1913).
[85] *Deut. med. Wochschr.*, **40**:1607 (1914). *See also* Kolmer and Toyama, *Am. J. Syphilis*, **2**:505 (1918).
[86] *Berl. klin. Wochschr.*, **54**:613 (1917); **55**:83 (1918); Jantzen, *Z. Immunitäts.*, **33**:156 (1921).
[87] The principles of the Meinicke reactions are considered in much detail by Epstein and Paul, *Arch. Hyg.*, **90**:98 (1921).

Bruck's Nitric Acid Reaction.[88]—This is based on the fact that the precipitate formed in syphilitic serum by nitric acid is less soluble in dilute nitric acid than the precipitate from normal serum. It agrees with the Wassermann reaction in about 70% of positive cases (Kolmer).

Formol Reactions.—Syphilitic sera form a solid gel when treated with formalin in concentrations which do not have this effect on normal sera.[89] This reaction is, however, far from constant, and often positive reactions are obtained with normal sera.[90]

Sachs-Georgi Reaction.—A specially prepared cholesterolized lipoid antigen from beef heart gives a much heavier precipitate in diluted inactivated syphilitic than in normal serum. This reaction takes advantage of the precipitation described in syphilitic serum when added to lipoidal "antigen" observed by ultramicroscopic methods by Jacobsthal, which is made visible to the naked eye under the conditions of this test. Active complement is not present in this reaction, which, according to Mackie, seems to depend on a different fraction of the serum proteins from the one responsible for the Wassermann reaction.[91] The precipitate consists chiefly of lipoidal matter,[92, 93] but is said to carry down with it the substance of the serum which gives the Wassermann reaction (Wassermann,[94] Taoka[95]).

Kahn Reaction.[96]—If an antigen suspension in salt solution which possesses the proper degree of instability, is added to normal serum, the particles at first dissolve, but if the serum is syphilitic a precipitate appears in a few minutes. This is much accelerated by agitation of the mixture, and the reaction appears to result from much the same physical conditions as the Sachs-Georgi reaction. It seems to depend on the globulins of the serum or spinal fluid, the albumin fraction being inactive.[97]

The Vernes Reactions.[98]—These are based on the observation that colloidal suspensions of inorganic substances, especially ferric hydrate, produce more flocculation in syphilitic than in normal sera. Colloidal suspensions of organic substances may exhibit the same differences.

Significance of the Flocculation Reactions

As seen in the above reactions the capacity of syphilitic serum (and spinal fluid) to produce flocculation with various colloidal or crystalloid precipitating reagents, is distinctly increased over the normal. The flocculated material is generally found on examination to contain both the serum protein, especially globulin, and the other agent involved

[88] *Münch. med. Wochschr.*, **64**:25 (1917).
[89] Gate and Papacostas, *Compt. rend. soc. biol.*, **83**:1432 (1920).
[90] Ecker, *J. Infectious Diseases*, **29**:359 (1921).
[91] Mackie, *J. Path. Bact.*, **26**:120 (1923).
[92] Niederhoff, *Münch. med. Wochschr.*, **68**:330 (1921).
[93] Klostermann and Wiesbach, *Deut. med. Wochschr.*, **47**:1092 (1921).
[94] *Berl. klin. Wochschr.*, **58**:193 (1921).
[95] *Kitasato Arch. Exp. Med.*, 5: No. 3, 1 (1922).
[96] Kahn, R. L., "Serum Diagnosis of Syphilis by Precipitation," Baltimore, Williams and Wilkins, 1925. Also Chapter 43 in Alexander's "Colloid Chemistry," 1928, Chemical Catalog Co., New York.
[97] Kendrick and Kahn, *J. Infectious Diseases*, **39**:202 (1926).
[98] *See* Kolmer, "Infection, Immunity and Serum Therapy," W. B. Saunders, Phila., 1923, p. 523.

in the flocculation. When the latter is a lipoid it seems to be the predominating part of the precipitate.[92] Whether this change in properties of the serum and spinal fluid depends on the increased proportion of globulins, or on changes in the quality of the globulins, or the presence of some new factor, possibly an antibody, is not known. That the mere increase of globulin cannot be responsible is indicated by the fact that many infections cause an increase in the proportion of globulin in the blood without causing it to give these reactions which are usually given by the serum of syphilitics, nor does the isolated globulin of normal serum give positive reactions.

It is probably of some significance that this new property is associated with the globulins,[99] for, as pointed out elsewhere, antibody properties in general are found associated with this fraction of the serum proteins. Mackie and Watson [100] find that treatment of serum with CO_2 throws out the agent which gives the Wassermann reaction in the precipitate, which consists chiefly of euglobulin with part of the pseudoglobulin, whereas the flocculation reactions are more active with the soluble fraction which contains mostly albumin and pseudoglobulin.[101]

Kolmer [98] belièves that the fundamental mechanism of complement fixation in syphilis is identical with these macroscopic colloidal flocculation reactions. The agglutinin and precipitin reactions with formed elements and proteins in solution are colloidal reactions even though the chemical nature of the antibody in the serum is unknown. It is entirely reasonable to assume that in the Wassermann reaction the same antibody-like substance is operative as in the various flocculation reactions, since in the former flocculation occurs which is invisible to the naked eye, but sometimes visible microscopically by the dark-field illumination method. In a general manner the tissue extracts yielding best results in the complement-fixation reaction also prove most sensitive in the macroscopic flocculation reaction, e.g., the cholesterolized alcoholic extracts.

Kolmer also calls attention to the fact that "the flocculating substance produced in syphilis and present in the blood and spinal fluid has a special effect upon lipoidal substances in colloidal suspension, and for this reason the substrate for most flocculating tests is an alcoholic

[99] Schuiringa and Kapsenberg, *Z. Immunitäts.*, **42**:300 (1925).
[100] *J. Hyg.*, **25**:176 (1926).
[101] Forssman [*Acta Path. Microbiol. Scand.*, **4**:198 (1927)] however, has observed that spinal fluids may give positive Wassermann reactions when they contain no globulin detectable by the Nonne or Pauli tests, whereas the Sachs-Georgi flocculation reaction is given only when the fluid contains demonstrable globulin, wherefore he concludes that these two reactions do not depend on the same agent.

extract of some tissue. The methods of Hermann and Perutz, Porges and Meier, Meinicke, Sachs and Georgi, Vernes, Kahn, etc., employ substrates of this kind or solutions of isolated lipoids, and the occurrence, sensitiveness, and practical value of the reactions in syphilis depend to a remarkable degree upon the method of preparing and diluting the extracts, as is true of the 'antigens' or extracts employed in the Bordet-Wassermann reaction. In the coagulation test of Hirschfeld and Klinger, a tissue lipoidal extract (the cytozyme) is regarded as essential for the production of thrombin by interaction with a serozyme (an albuminoid) and a calcium ion, and the reaction is based upon the observations that syphilitic serum inhibits coagulation by its effect upon the lipoidal extract (cytozyme), probably one of inactivation by flocculation."

The Active Agent in the Syphilis Reactions

Despite the abundant evidence on the nature of the active substances at play in the complement fixation and specific flocculation tests cited in the foregoing paragraphs, we can say nothing more definite than that *the proportion and character of serum globulins and lipoidal colloidal suspensions are altered in syphilis in some unknown way so that their stability is greatly reduced and hence they tend to flocculate and to adsorb other colloids, including among them the serum colloids which exhibit the function of "complement."*

But, on the other hand, Breinl [102] points out the fact that positive Wassermann reactions are given in syphilis by such widely differing media as serum, cerebrospinal fluid, milk, and the aqueous humor of the eye. This seems difficult to reconcile with the theory that the reaction is dependent on the physico-chemical properties of the medium, and more in harmony with the view that some specific substance must be present, presumably of the nature of a true immune antibody, as Wassermann [94] still maintained in his latest papers. This is also supported by the observations of Landsteiner, Sachs, Klopstock, and Weil on the occurrence of antibodies for lipoid-protein antigens. In further support of this view is Breinl's observation that the complement-binding agent of syphilitic sera may be bound to organ cells and removed from the serum without appreciably decreasing the amount of globulin or lessening the capacity of this serum to produce the precipitation reaction of Bruck which depends on the serum globulins. This, however, might be explained by Forssman's results, which indicated that

[102] *Z. Immunitäts.*, **29**:463 (1920).

the active substance in syphilitic serum is not the globulin itself but an associated lipoid which probably is not a true immune antibody.

Gloor and Klinger [103] found that removal of the globulin from syphilitic serum does not remove the positive Wassermann reaction.[104] Normal sera which have been made Wassermann positive by such procedures as shaking or treatment with fine suspensions such as kaolin, however, lose this artificial reactivity on removal of the globulin. These observations suggest that while alterations in the colloidal state of the globulins may cause a serum to become Wassermann positive, yet the reaction in syphilis depends at least in part on something else besides the globulin. They agree with the view of Sachs [105] who suggests that the reaction involves first the lipoids of the serum and the antigen, and that the specific behavior of the syphilitic serum depends on a primary alteration of the lipoids from which results a secondary globulin alteration which modifies the degree of its dispersion. In this connection may be recalled the fact brought out by Chick that euglobulin is apparently a lipoid-globulin compound.

Nathan [106] found that artificially activated normal sera which give positive Wassermann reactions do not behave like syphilitic sera in the Sachs-Georgi reaction. He therefore agrees with the foregoing authors in the opinion that the changes produced artificially in normal serum by physico-chemical means do not correspond to the changes which are characteristic of syphilis. The artificial positive reactivity of sera, which depends merely on alterations in the globulin, is thermolabile; the thermostable alteration characteristic of syphilis probably depends on changes in the lipoids, either alone or in combination with the serum proteins.

Epstein and Paul [106a] observe that in all the syphilitic reactions with lipoid antigens, the lipoids are presented in the form of a complex emulsion-suspension colloid, which is unstable and tends to flocculate spontaneously on standing, for a large proportion of the particles are on the border-line between coarse suspensions and true colloidal dispersion, and are readily aggregated into coarser units. In such a reaction as that of Meinecke there is apparently a discharge of the electronegative lipoid phase by the Na ions of the NaCl, while the protein phase of the serum takes a positive charge. This discharge of

[103] *Z. Immunitäts.*, **29**:435 (1920).

[104] On the other hand, Stern [*Klin. Wochschr.*, **2**:1411 (1923)] found that the euglobulin precipitated from syphilitic serum by electrolysis gives the specific Wassermann reaction, the remaining proteins giving negative reactions. [Also *Z. Immunitäts.*, **39**:293 (1924).]

[105] *Kolloid-Z.*, **24**:123 (1919).

[106] *Z. Immunitäts.*, **29**:562 (1920).

[106a] *Arch. Hyg.*, **90**:98 (1921).

the lipoid phase leads eventually to flocculation. Normal serum behaves as a protective colloid which preserves the colloidal stability of the lipoid emulsion which constitutes the "antigen," whereas syphilitic serum not only fails to protect but actually augments the flocculation. Apparently, then, syphilitic infection produces some distinct changes in the electrical charge or surface tension of the serum colloids, which as yet have not been determined.[107] These authors, like many others, consider the flocculation reactions to be related to the Wassermann reaction in that both depend on condensation of the disperse particles of the lipoid phase of the "antigen"; in the Wassermann reaction the flocculation may not be visible, but nevertheless occurs to sufficient degree to lead to complement binding by the aggregates, whereas in the flocculation reactions there is a much greater loss of dispersion of the lipoid colloids.

Recapitulation

The Wassermann reaction apparently is not fundamentally the same thing as the complement-fixation reaction of Bordet and Gengou, even although it is demonstrated in the same way by fixation or inhibition of the complement which hemolyzes red corpuscles sensitized with specific hemolytic antibody. In the Wassermann reaction the "antigen" used to bring about the fixation of complement is not a specific antigenic protein, as in the true complement-fixation reaction, but may consist of varied lipoidal mixtures prepared from many sorts of tissues and having nothing to do with either the disease syphilis or the organism which causes it. This "antigen" seems to be a complex mixture of crude tissue lipoids, in which lecithin predominates and a small proportion of cholesterol is important, pure lecithin being inactive. The existence of any specific agent in the crude mixture has not been established. Colloidal suspensions which do not contain lipoids are not effective. Probably the activity of these "antigens" depends largely on the state of colloidal dispersion of the lipoidal emulsion. In the sense of being able to incite antibody formation when injected unaccompanied by proteins into animals, these lipoidal mixtures are not antigenic.

There is no conclusive evidence that an antibody is involved in the Wassermann reaction. Although the immunization of rabbits with crude lipoids mixed with swine serum causes their serum to become strongly Wassermann positive, this does not prove that a similar immunization

[107] During the course of the reaction there is a lowering of surface tension, greater in positive than in negative systems. [Parrino and Brancato, *Boll. ist. sieroterap. Milanese*, 4:27 (1925)].

to lipoid-protein mixtures is responsible for the Wassermann reaction in man. Nevertheless it is a strong argument in favor of the hypothesis that this reaction depends on an autoimmunization with the formation of antilipoid immune substances.

The serum of syphilitics which gives a positive Wassermann reaction seems to owe this property to quantitative and qualitative changes in the globulins. These are much increased in amount, and are so altered, possibly through combination with lipoids, that they have a greatly decreased stability, whereby they are much more readily flocculated by slight chemical and physical changes in the fluid medium in which they are dissolved, or have an increased capacity to facilitate the flocculation of the colloidal suspension of lipoids used as antigen, the resulting colloidal aggregates tending to adsorb complement. Normal serum may be made to give positive Wassermann reactions if submitted to shaking, slight heating, slight changes in pH or salt concentration, etc. Such artificial activation, however, presents distinct differences from the activation produced in the serum by syphilis.

There is much reason to believe that lipoids combined with proteins play as important a part in the rôle of the "amboceptor" as the lipoids play in the "antigen" itself, the reaction depending on the physical state of these two agents, which is in part dependent on their chemical nature. In view of the abundant evidence now available concerning the nature of the active substances which are responsible for the Wassermann reaction and for the numerous, more or less specific flocculation tests given by syphilitic serum, these latter reactions are of interest here as showing that there must be considerable changes in the colloids of the blood serum in syphilis, although they are for the most part inferior in clinical diagnostic value to the refined Wassermann reactions.

CHAPTER IX

HYPERSENSITIVENESS—ANAPHYLAXIS—ALLERGY

One of the most spectacular phenomena discovered in immunity is that of hypersensitivity to foreign proteins. It is a startling fact that a guinea pig, which can tolerate many cubic centimeters of such a protein mixture as horse serum in a single dose, will be almost immediately killed by as little as 0.01 cc. of this same serum provided a similar or even much smaller amount has been injected into it ten days or more previously. The character of the death with violent convulsions, perhaps within a minute of the time the injection is made, makes this observation all the more dramatic. The general features of this process are covered fully in numerous text books and reviews [1] and will not be gone into extensively here. The main facts which should be presented to serve as a basis for the discussion of the chemical features of the process, are as follows:

General Features of Anaphylaxis

(a) Any protein capable of serving as an antigen in other immunological reactions may be used to produce the typical anaphylaxis reaction, and, as far as now established, nothing else will do it. The amount of protein necessary to produce the reaction is extremely minute. With crystallized egg albumin I have succeeded in sensitizing guinea pigs with a single dose as small as one twenty-millionth of a gram, and fatal sensitization has resulted from one-millionth of a gram. Within certain limits large doses are less effective in sensitizing guinea pigs than small, e.g., one milligram of most proteins will usually be much more effective than one hundred milligrams.

(b) To produce intoxication in guinea pigs thus sensitized, somewhat larger minimum doses are required, about one-twentieth or one-tenth milligram by intravascular injection being minimum lethal doses

[1] Doerr, *Ergebn. Hyg. Bakt. Immun. Therap.*, 5:71 (1922); Coca, "Tice's Practice of Medicine," New York, Prior and Co., 1920, 107; Wells, *Physiol. Rev.*, 1:44 (1921), and the following chapters in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1929; LXXIII, "Anaphylaxis and Anaphylactoid Phenomena," by H. T. Karsner; LXXIV, "The Technique of Experimentation in Anaphylaxis," by W. H. Manwaring; LXXV, "Atopy," by A. F. Coca.

with most of the purified proteins tested. Intraperitoneal injections require doses about fifty times as large to secure equally severe reactions, much depending on the solubility of the protein in the peritoneal fluid.[2] Subcutaneous injections are ordinarily absorbed too slowly to produce any serious effects.[3]

(c) The proteins concerned must be foreign to the circulating blood of the injected animal, but they may be tissue proteins of the same animal (e.g., placenta elements, organ extracts, lens proteins) which are not normally present in its blood. Indeed, it has been claimed that by injecting a guinea pig with the dissolved lens of one eye it will become sensitized so that it will react to a subsequent injection of the lens from the other eye. In general, tissue proteins are less active antigens than the proteins of the blood, lymph, and secretions, perhaps because less soluble, but it is reported that even keratins may produce anaphylaxis when dissolved.[4]

(d) The symptoms and anatomical changes are quite the same in the same species of animal, no matter what sort of protein is serving as the antigen in producing the anaphylaxis reaction, although the effects in different species are characteristic for each species. This fact indicates that the anaphylactic manifestations depend always on a similar sort of immunological reaction, and not on the sort of antigen employed.

(e) An interval of time of at least 7 days, and better 10 or more days, should elapse between the first or sensitizing and second or intoxicating injection, the strongest reactions usually being obtained after the third week, the sensitization slowly decreasing but often persisting throughout life. The offspring of sensitized female guinea pigs may be sensitive for some time after birth, but gradually lose this passive sensitivity.

(f) After recovering from a severe, non-fatal reaction the animal is usually refractory to another dose of the same protein, as if the antibodies responsible for the condition of anaphylactic sensitization had been exhausted. Sensitivity may return after a considerable period, apparently through the formation of new antibodies.

(g) The blood of a sensitized animal usually contains substances (antibodies) similar to those responsible for the production of the typical reaction that occurs on re-injection of the antigen. This is shown

[2] Wells and Osborne, *J. Infectious Diseases*, 14:377 (1914).
[3] Lewis, J. H., *J. Am. Med. Assoc.*, 76:1342 (1921).
[4] Krusius, *Arch. Augenheilk., Suppl.*, 47:47 (1910); Clough, *Arb. kais. Gesundheitsamte* 31:431 (1911).

by injecting the blood or serum of a sensitized guinea pig into a normal guinea pig, for this animal now is made sensitive within a few hours, instead of after a wait of several days as in active sensitization. This passive sensitization is transitory, disappearing within thirty days.[5] In general, any serum which contains precipitins for a given protein will, when injected into a normal guinea pig, render it passively sensitive to this same protein. As the capacity of a serum to induce passive anaphylaxis is in direct proportion to its precipitin content, it is generally believed that anaphylactic sensitization depends on an antibody which is identical with precipitin, and probably also with the complement fixation antibody.[6]

(h) Not all animals are equally susceptible to anaphylactic intoxication, nor do they behave in the same way. Guinea pigs are most readily affected, the reaction in them being characterized by bronchial spasm leading to death from acute asphyxia. In dogs the most marked effect is seen to be a severe congestion of the entire splanchnic area, often with a hemorrhagic enteritis. In rabbits there is an acute dilatation of the right heart from spasm of the pulmonary vessels.[7] Other symptoms may be observed which all seem to depend upon contraction of non-striated muscle, and this is the basis of most of the manifestations of anaphylaxis. Man may exhibit any or all of the symptoms seen in other animals, sometimes reacting like the dog with marked hepatic and splanchnic congestion;[8] often, especially in asthmatics, developing bronchial spasm,[9] but more often showing cutaneous lesions as the most marked symptoms.[10] Except for a small proportion of the population, however, man seems ordinarily not to be very reactive, and monkeys also are inactive in respect to anaphylaxis,[7] as well as to antibody formation in general.[11]

(i) Non-striated muscle from a sensitized animal will, if removed from the body and placed under suitable conditions, be found to give strong contractions when brought in contact with even very dilute solutions of the specific antigen. Uterine muscle (from virgin guinea pigs) is most commonly used, but contraction of arterial, intestinal and bronchial walls has been observed.

[5] Kellaway and Crowell, *Brit. J. Exp. Path.*, 4:255 (1923).
[6] Friedberger and Scimone, *Z. Immunitäts.*, 36:386 (1923).
[7] *See* Drinker and Bronfenbrenner, *J. Immunol.*, 9:387 (1924).
[8] Dean, *J. Path. Bact.*, 25:305 (1922).
[9] Huber and Koessler, *Arch. Int. Med.*, 30:689 (1922).
[10] For a discussion of the cutaneous reactions *see* Kolmer, *Bull. Johns Hopkins Hosp.*, 28:163 (1917).
[11] Zinsser, *Proc. Soc. Exp. Biol. Med.*, 18:57 (1920).

The Definition of Anaphylaxis

Much confusion exists in the literature because of loose use of the term anaphylaxis to cover a multitude of conditions, not all of which are true antigen-antibody reactions, to which it seems desirable in the present state of our knowledge to restrict the use of the term anaphylaxis. Not all sudden attacks of dyspnea and convulsions in injected guinea pigs, or of cardiac syncope or cutaneous irritation in man, are manifestations of anaphylaxis in this sense, or are even related to this condition except in a greater or less similarity of these particular symptoms. Even distention of the lungs in guinea pigs, circulatory disturbances in rabbits, or splanchnic congestion in dogs, following intravascular injections of foreign materials, are not necessarily proof of a true anaphylactic reaction, for each and all may be produced by materials or under conditions that surely do not involve the same processes that are responsible for the reaction which occurs when a small quantity of an otherwise harmless protein is injected into an animal that has been previously prepared by injection of the same protein. We must grant that this last is true anaphylaxis, and must demand that the term anaphylaxis be limited to conditions that result from a similar mechanism within the body of the reacting animal. At least the following criteria must be met:

1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in non-sensitized animals.

2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.

3. It should be possible to demonstrate typical reactions in the non-striated muscle tissue of the sensitized animal.

4. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.

5. After recovery from anaphylactic shock there should be exhibited a condition of specific desensitization to the same antigen under proper conditions.

6. In addition to the above, it is usually, but not always, possible (a) to demonstrate passive sensitization with the serum of sensitized animals; and (b) to demonstrate amelioration or prevention of the bronchial spasm in guinea pigs by proper use of atropine and epinephrine.

We know that the foregoing conditions are fulfilled in the typical anaphylactic reaction with soluble protein antigens. Reactions produced by substances not coming within this class cannot properly be considered as true anaphylactic reactions unless they can be demonstrated to exhibit the same phenomena. The term *allergy* is broad enough to cover all those other manifestations of altered reactivity and to indicate their relationship to one particular form of allergy, anaphylaxis.[12]

Allergy (*ergia,* "reactivity" and *allos,* "altered") is the term introduced by von Pirquet which Doerr adopted to signify any altered degree of reactivity, whether increased or decreased from the usual condition, and whether depending upon antigen-antibody reactions or upon the effects of entirely non-antigenic substances, such as hypersusceptibility to light and drugs. Although Coca [13] and others have suggested various modifications of the terminology used in discussing these processes of altered sensitivity, it is agreed by many writers that *the term anaphylaxis should be restricted to the condition of hypersensitivity to definitely antigenic substances,* according to the limitations specified above.[14]

Nature of the Antigens (Anaphylactogens, Allergens)

These seem to be entirely the same as the antigens effective in producing all the other immunological reactions, and they are fully discussed in Chapter II. In brief, all typical soluble proteins will produce typical anaphylaxis reactions, and there is still doubt as to their production by any other material. The products of the hydrolysis of proteins are also ineffective.[15] Since no positive results can usually be obtained with most of the fractions of protein cleavage, including digestion mixtures of various sources, but containing usually all the cleavage products from proteoses through peptones and polypeptids to amino acids,[16] it is not easy to accept the statement that anaphylaxis can be produced by the relatively simple synthetic polypeptids, even one containing 14 molecules of leucine and glycine (Abderhalden),[17] still

[12] *See* review by Doerr, "Die Idiosynkrasien," in Mohr and Staehelin's "Handbuch der inneren Medizin," 2nd Edition, 1926, p. 448.

[13] Coca and Cook, *J. Immunol.,* 8:163 (1923). *See also* Doerr, *Arch. Dermatol. Syphilis,* 150:509 (1926).

[14] The hypersensitivity to certain bacteria manifested by such allergic phenomena as the tuberculin reaction, seems to be fundamentally distinct from true anaphylaxis [*See* Zinsser and Tamiya. *J. Exp. Med.,* 44:735 (1926)].

[15] Fink, *J. Infectious Diseases,* 25:97 (1919). However, Landsteiner reports positive results with the products of peptic digestion of egg globulin [*Proc. Soc. Exp. Biol. Med.,* 23:540 (1926); 25:666 (1928)].

[16] The increased reaction of an intestinal segment observed to follow a second dose of cysteine an hour or so after a first dose [Voegtlin and Dyer, *J. Pharm. Exp. Therap.,* 29:105 (1926)] seems not exactly comparable to anaphylactic hypersensitivity.

[17] *Z. physiol. Chem.,* 81:315 (1912).

less the positive results of Zunz [18] with much simpler polypeptids (3-5 glycylglycine).

There still remains no satisfactory proof that anything except proteins can act as anaphylactogens. Even some definite antigens, whose protein nature is doubtful, are not anaphylactogens in the strict sense, namely, true soluble toxins, despite the fact that they do produce a certain type of hypersensitiveness.[19] While many attempts have been made to establish the antigenic activity of lipoids by means of the precipitin and complement fixation reactions, the lack of solubility of the lipoids has made them unsuitable for anaphylaxis studies. Lipoids and carbohydrates have not yet been demonstrated to be capable of sensitizing animals to themselves, although, as with other immunological reactions, mixtures of lipoids with foreign proteins may produce sensitization to these mixtures, and certain modifications of the specificity of the reactions may be found dependent on the lipoids.[20] Klopstock [21] reports that after desensitization of such animals with the specific foreign sera, reactions are still given to the lipoids. But since in animals sensitized with such lipoid-protein mixtures the anaphylactic antibodies cannot be removed by injecting the lipoids alone (desensitization), Doerr [22] maintains that anaphylaxis is essentially solely a protein-antiprotein reaction, and questions the existence of true lipoid anaphylaxis. Probably the situation with lipoids is similar to that with bacterial carbohydrates, for these will shock guinea pigs sensitized with whole bacterial antigens, although the isolated carbohydrate will not sensitize.[23] Certainly as yet the antigenic capacity of lipoids independent of proteins has not been established (see also p. 56).

Drug Hypersensitivity.—On the other hand, drugs and chemicals of many sorts may produce both local and systemic reactions that have been interpreted as anaphylaxis. Especially is this true of mercurials, salvarsan and other arsenicals, iodin and bromin and compounds thereof; also innumerable alkaloids (e.g., quinine, atropine), synthetic organic compounds (salicylates, antipyrine) and various vegetable oils, resins, etc.[24] (The evidence concerning the reactions

[18] *Biochem. J.*, **10**:160 (1916); *J. physiol. path. gén.*, **17**:449 (1917); *Arch. intern. physiol.*, **15**:179, 192 (1919); *Bull. acad. roy. med. Belg.*, **3**:426 (1923).

[19] *See* Coca, Tice's "Practice of Medicine," New York, Prior and Co., 1920, 107.

[20] *See* Henning, *Z. Immunitäts.*, **55**:19 (1928).

[21] A. Klopstock, *Z. Immunitäts.*, **48**:97 (1926); full review on lipoid anaphylaxis.

[22] Doerr and Blyer, *Z. Hyg.*, **106**:371 (1926).

[23] Tomcsik, *Proc. Soc. Exp. Biol. Med.*, **24**:812 (1927); Lancefield, *J. Exp. Med.*, **47**:843 (1928); Tomcsik and Kurotchkin, *ibid.*, p. 379.

[24] Primrose dermatitis in susceptible persons is produced by a non-protein, crystallizable substance, *primin*, according to Bloch and Karrer [*Vierteljahrsschr. naturforsch. Ges. Zürich*, **72**: Beibl. 13, p. 1 (1927)].

produced by these substances has been thoroughly reviewed by Coca,[25] Doerr,[26] and Biberstein.)[27]

Wolff-Eisner advanced the hypothesis that drug reactions may be produced by the blood or tissue proteins altered by the chemical so that they are as foreign proteins to the injected animal. This hypothesis has gained weight especially from the observations of Landsteiner on the antigenic capacity of proteins compounded with various non-protein radicals, and discussed elsewhere (pp. 36, 95). He found that the serum proteins of an animal could be so altered by addition of simple chemical radicals that the artificial compound protein when injected into this same species of animal incites the formation of precipitins for this compound protein. In view of the close relation if not identity of the antibodies responsible for the various immunological reactions, it is fair to transcribe the above results to anaphylaxis, and Swift [28] has obtained some suggestive results with salvarsanized guinea pig serum. Landsteiner [29] tested an artificial azoprotein (diazotized para-arsanilic acid combined with protein) and found that it sensitized guinea pigs not only to itself but to different proteins united to the same azo-compounds. Furthermore, injection of simple non-protein compounds containing the same or similar radicals, desensitized animals to the compound proteins, although such non-protein compounds were not capable of sensitizing either to themselves or to the compound proteins. Klopstock and Selter [30] even found it possible to combine diazotized atoxyl with guinea pig proteins and with them produce anaphylactic reactions in guinea pigs. Subcutaneously injected, diazotized atoxyl will combine with the tissue proteins to function as an antigen. Samson and Götz [31] claim to have obtained true anaphylactic sensitization and reaction in guinea pigs with amidopyrine mixed with guinea pig blood.

Prausnitz and Küstner found that in some cases of human idiosyncrasy to protein agents, the serum of the hypersensitive individual injected into the skin of a normal guinea pig renders this area of skin specifically hypersensitive to the same agent after 24 hours' incubation. Biberstein [32] has tested out this method extensively with non-protein

[25] Coca, "Tice's Practice of Medicine," New York, Prior and Co., 1920, 107; and chapters in "The Newer Knowledge of Bacteriology and Immunology," Univ. of Chicago Press, 1928, LXXIV, LXXV.
[26] *Ergebn. Hyg. Bakt. Immun. Therap.*, 5:71 (1922).
[27] Biberstein, *Z. Immunitäts.*, 48:297 (1926).
[28] *J. Am. Med. Assoc.*, 59:1236 (1912).
[29] *J. Exp. Med.*, 39:631 (1924).
[30] *Klin. Wochschr.*, 6:1662 (1927).
[31] *Z. ges. exp. Med.*, 52:121 (1926).
[32] *Loc. cit.*,[27] full review. *See also* Gay and Chant, *Johns Hopkins Hosp. Bull.*, 40:265 (1927).

agents and obtained local passive transfer to guinea pigs of human sensitivity to mercury, bismuth, salvarsan and pyramidon, but not with numerous other non-protein substances to which patients were hypersensitive. This reaction of passive sensitization seems to indicate that at least some, if not all, the instances of specific cutaneous drug hypersensitivity do depend on the occurrence of a true anaphylactic sensitization to compound proteins. Probably many of the systemic reactions observed after *intravenous* injections of arsenicals and other substances are not anaphylactic but *anaphylactoid,* and dependent upon capillary thrombosis, agglutination emboli, or capillary toxicity, after the order of the results obtained by Karsner and Hanzlik.[33]

The Nature of the Immune Body (Anaphylactin, Anaphylaxis Reaction Antibody, Allergin, Sensibilizin, Sensitizin)

The demonstration that anaphylactic sensitization can be transferred to a normal animal by injection of blood or serum from a sensitized animal, has been generally accepted as establishing anaphylaxis as a typical antigen-antibody reaction. Doerr and Russ added important support to this view by showing that the potency of a serum in conferring passive sensitization is in direct proportion to its content in precipitins. Zinsser states that "The principle that anaphylaxis depended directly on the meeting of the antigen with its specific antibody has never been seriously questioned since that time." Three main questions have remained unsolved, however, namely: a, Is the sensitizing antibody the same as the precipitin or the complement-fixing antibody? b, Where does the reaction of antigen and antibody take place? c, How does this reaction produce the manifestations of anaphylactic shock?

The Relation of Precipitin to the Sensitizing Antibody.—In Chapter IV has been discussed at length the question as to the identity of the immune antibodies in different reactions, and the evidence has been given for and against the "unitarian" hypothesis.

Anaphylaxis apparently comes into the class of "proteolytic" immune reactions, and the identity of the sensitizing antibody and the precipitin has been strongly supported. Several observers have found that the relative proportion of precipitin and sensitizing antibody runs parallel in immune sera (Friedberger, Doerr and Russ) and I have myself observed, in studying the development of antibodies in animals immunized to vegetable proteins, that the precipitins appear together with

[33] *J. Pharm. Exp. Therap.,* 14:229, 379, 425, 449, 463, 479 (1919); 23:173 (1924); Hanzlik, *J. Am. Med. Assoc.,* 82:2001 (1924).

the capacity to confer passive sensitization.[34] Richard Weil [35] added much support to this contention by finding that the precipitate obtained when immune serum reacted with the specific protein, and washed free from serum, conferred passive sensitization to the specific antigen when injected into guinea pigs, if the proportion of antiserum and antigen had been suitably selected in producing the precipitate. The precipitates produced in the precipitin reaction contain both antigen and antibody, since they sensitize both actively and passively, and Weil believed that both antigen and precipitin can be separately extracted from such precipitates by suitable procedures. Although Coca [36] was unable to repeat Weil's experiment with washed precipitin-antigen precipitate, yet he did secure further evidence that precipitin and sensitizing antibody are identical.

Doerr and Hallauer [37] separated into fractions the proteins of immune sera by electro-dialysis and electro-ultrafiltration, and found that the precipitin and the anaphylactin were regularly found together in the different fractions. Otto and Iwanoff [38] obtained much less regular relations. Apparently as yet the methods available for fractionating serum proteins are not sufficiently refined to give valuable evidence as to the identity or non-identity of immune bodies. However, the identity of the anaphylactic with other antibodies is further supported by the fact that the serum of animals immunized with bacteria will give precipitin, complement fixation, and passive sensitization reactions with the isolated specific carbohydrates of these bacteria, which carbohydrates are not themselves antigenic.[39]

If we grant the identity of precipitin and sensitizing antibody,[40] one must consider the relation of both to the complement-fixing antibody, which numerous observers have maintained is identical with the precipitin.[41] The assumption of identity of the sensitizin and the complement fixation antibody is the basis of Friedberger's classical experiments on the production of a poison, supposed by him to be responsible for anaphylaxis, by digestion of sensitized proteins with complement. This topic will receive consideration later.

The Site of the Reaction.—The earlier view that anaphylaxis resulted from reaction of antigen and antibody in the circulating blood

[34] Lake, Osborne and Wells, *J. Infectious Diseases*, **14**:364 (1914).
[35] *J. Immunol.*, **1**:19 (1916).
[36] Coca and Kosakai, *J. Immunol.*, **5**:297 (1920).
[37] *Z. Immunitäts.*, **47**:363 (1926); **51**:463 (1927).
[38] *Z. Immunitäts.*, **54**:496 (1928).
[39] Tomcsik and Kurotchkin, *J. Exp. Med.*, **47**:379 (1928).
[40] Manwaring reports a lack of correspondence of precipitin and anaphylactin in dogs [*J. Am. Med. Assoc.*, **86**:1271 (1926); *J. Immunol.*, **13**:59 (1927); **15**:351 (1928)].
[41] Zinsser, *J. Exp. Med.*, **18**:219 (1913).

was shaken by the observation that in passive sensitization of guinea pigs by injection of serum containing antibodies, there is a latent period of at least four hours before the animal becomes sensitive,[42] and that simultaneous injection of antigen and antiserum produces no effects.[43] These facts are difficult to explain if antigen and antibody react in the circulating blood, but fit in well with the hypothesis that this latent period is required for the antibody to enter the cells and render them sensitive. The humoral reaction hypothesis was rendered untenable as a complete statement of anaphylaxis by the observation of Schultz that isolated segments of intestine from sensitized animals reacted to the specific antigen by contraction, and by the development and refinement of this principle especially by Dale and Weil. It has now been established that isolated non-striated muscle tissue, taken from a sensitized animal and washed as free as possible from blood, will give strong contractions immediately it is brought into contact with even very dilute solutions of the specific antigen, and only with the specific antigen.

Another form of evidence of the non-humoral nature of anaphylaxis is furnished by transfusion experiments (Manwaring, Pearce and Eisenbrey, Weil and others) which show that the blood of a sensitized animal can be replaced by the blood from a non-sensitized animal without impairing the sensitivity of the animal as a whole or of its isolated tissues. This form of experiment is particularly convincing when passive sensitization is employed with serum of known sensitizing power, since then it is possible to know surely that the transfused animal cannot have a sensitizing dose of antibodies left in its circulating blood (Coca,[44] von Fenyvessy and Freund[45]). Furthermore, Doerr and Pick have observed that even after all demonstrable antibodies have disappeared from the circulating blood in the rabbit, fatal anaphylactic shock may be produced.

Not only do we have all this evidence of the cellular site of the anaphylactic reaction, but in addition there is abundant evidence that the presence of free antibodies in the blood interferes with the anaphylactic reaction (Weil),[46] presumably by binding the antigen before it can reach the "sessile" receptors within the cells. This is the explanation of the fact that repeated injection of guinea pigs with the antigen renders them immune rather than hypersensitive, and that a single large dose

[42] A similar latent period is required for *reversed anaphylaxis; i.e.*, injection of specific antibody into an animal previously injected with antigen. [Opie and Furth, *J. Exp. Med.*, 43:469 (1926).]

[43] *See* Doerr and Bleyer, *Z. Hyg.*, 106:371 (1926).

[44] *Z. Immunitäts.*, 20:622 (1914).

[45] *Z. Immunitäts.*, 22:59 (1914).

[46] *J. Med. Res.*, 30:299(1914).

of antigen sensitizes less successfully than a small dose of the same antigen.

It may safely be said that at least the essential features of acute anaphylactic shock depend upon intracellular reaction between antigen and antibodies that have been withdrawn from the circulating blood and fixed within the cells. Whether the reaction which presumably occurs between antigen and the free antibodies in the circulating blood also plays any part whatever in anaphylaxis, is still undetermined.[47]

On the other hand, there is no doubt that antigen-antibody reactions do produce, at least *in vitro*, substances that are eminently injurious, especially upon intravascular injection, and it seems reasonable indeed to believe that such substances may be produced in the typical anaphylaxis reaction and play some part in it, even if we grant that the typical acute anaphylactic shock depends on reaction within certain tissue cells.

"Anaphylatoxin" Formation and Its Relation to Anaphylaxis

For a time in the history of the study of the anaphylaxis reaction the cause of the intoxication clearly seemed to lie in the cleavage of foreign proteins into toxic elements by the enzymes of the blood or the tissues. This "anaphylatoxin" hypothesis fitted so well most of the facts then known, and was so perfectly logical, that it seemed almost inevitable; yet at the present time it appears to be untenable, in the face of existing evidence, as the final explanation of anaphylaxis. It rested fundamentally upon three sets of observations. First, the demonstration by Vaughan that from almost any protein a highly poisonous substance can be produced by cleavage under suitable conditions, and that this substance produces effects in animals much resembling the phenomena of anaphylactic shock or subacute anaphylactic intoxication. Second, the similarity of the effects produced by intravenous injection of Witte's "peptone" and other products of protein cleavage, to those of anaphylaxis. Third, the demonstration by Friedemann and Friedberger of the highly poisonous character of the products of reaction *in vitro* of antigen, antibody and complement. Taken together, these observations seemed to indicate plainly enough that anaphylaxis was a typical case of poisoning with products of specific proteolysis of antigens by serum complement after sensitization of the antigen by the specific antibody. This brought anaphylaxis into the same class with the cytolytic reactions accomplished by the action of serum complement upon cells sensi-

[47] *See* Kritchevsky and Birger, *J. Immunol.*, 9:339 (1924).

tized by specific immune antibody, the poisoning presumably being due to the products of proteolysis by complement.

Friedberger showed that toxic products were formed when precipitates, resulting from the reaction of antigen and specific precipitin (or bacteria or other cells that had been sensitized with the specific cytolytic amboceptor), were digested for a suitable period with serum complement. Also, that the toxic products produced symptoms resembling those of anaphylaxis when injected intravenously into animals, and that these symptoms were the same irrespective of the nature of the antigen used, which is one of the characteristic features of anaphylaxis as well as of Vaughan's poisonous products of protein cleavage. Furthermore, too long digestion, or excessive sensitization, failed to produce the "anaphylatoxin," which fact supported the view that the poison represented an early stage in protein cleavage, which was destroyed by further digestion.

It was soon found, however, that this so-called anaphylatoxin formation was not a specific result of antigen-amboceptor-complement reaction, as identical toxic effects are produced with normal serum that has been digested with complement in the presence of absolutely inert but finely divided insoluble materials, such as kaolin, barium sulfate and agar. Jobling and Peterson [48] found that merely extracting the lipoids from normal serum and then letting it digest itself resulted in the same type of toxicity, and they agree with Bronfenbrenner [49] in attributing all the positive results in this type of experiment to a removal or inhibition of antienzymes present in normal serum, so that proteolysis then sets in.

Jobling and his co-workers maintained that in anaphylactic shock the blood shows the following changes: (a) the instantaneous mobilization of a large amount of non-specific protease, (b) a decrease of antiferment, (c) an increase in non-coagulable nitrogen of the serum, (d) an increase in amino acids, (e) a primary decrease in serum proteoses. They conclude that the intoxication is brought about by the cleavage of serum proteins (and proteoses) through the peptone stage by a non-specific protease and that the specific features lie in the rapid mobilization of this ferment and the colloidal serum changes which bring about the change in antiferment titer. The resulting poison is believed to be derived from the serum proteins of the animal rather

[48] *J. Exp. Med.*, 22:401, 590 (1915).
[49] *J. Exp. Med.*, 21:480 (1915).

than from the antigen, and presumably is the same as or similar to Friedberger's anaphylatoxin.

The mere clotting of blood or plasma may also produce changes which render it highly toxic, even for the animal from which it came, with the same anaphylaxis-like manifestations following intravenous injection that are produced by the toxic sera in the typical anaphylatoxin experiment.

Shortcomings of the Anaphylatoxin Hypothesis.—Despite the undeniable fact that blood serum may be made highly toxic when digested with complement under various conditions, and that the symptoms which follow intravascular injection of such toxic sera resemble those of anaphylactic shock in many particulars, nevertheless the responsibility of such a production of toxic products of protein cleavage for the explanation of anaphylactic shock, fails of proof. Some of the points that have been advanced against this so-called humoral anaphylatoxin theory are the following:

1. It does not fit with the latent period required after injection of the serum of a sensitized animal into a normal animal before the latter becomes sensitive (passive sensitization).[50]

2. Complement is not essential for anaphylactic shock, since animals deprived of free complement in the circulating blood may still give anaphylactic reactions. Here one may suggest the presence of intracellular or reserve complement.[51]

3. All attempts to prove that complement is a proteolytic ferment have so far failed. In the production of "anaphylatoxin" by various procedures there is no chemical evidence of protein cleavage, but its formation is associated with the presence of larger colloidal aggregates than those in normal serum and visible with the ultramicroscope.[52] As further evidence that anaphylatoxin formation is not the result of digestive proteolysis is the observation of Kopaczewski that anaphylatoxin is produced in serum in contact with gels at 0° C.

4. "Anaphylatoxin" activity has been produced in serum by acting on the serum with various colloids and suspensoids in the absence of complement, in the absence of antigen, and in the absence of antibody.

[50] Friedberger [*Klin. Wochschr.*, 4:1823 (1925); *Z. Immunitäts.*, 53:39 (1927)] has reported that if passive sensitization is produced by injecting the serum of an immunized guinea pig into a normal guinea pig, a reaction can be produced without the usual latent period required when the antibody is in a foreign serum, but this has failed of confirmation by others.

[51] Friedberger still defends the conception of anaphylaxis as the result of the formation of a poison through the action of complement, and has reported that if a serum which contains precipitins for more than one antigen is used to produce passive sensitization by injection into animals, it only sensitizes to such proteins as give loose precipitates which bind complement, and not to proteins which produce a more finely divided precipitate which does not bind complement [E. Friedberger and V. Scimone, *Z. Immunitäts.*, 36:386 (1923)].

[52] Dale and Kellaway, *Trans. Royal Soc. (London)*, 211B:273 (1922).

On the other hand, if antigen and the specific antibody are injected simultaneously into the opposite jugular veins of a guinea pig, the animal shows no evidence of intoxication.

5. When there is an abundance of free antibodies in the circulating blood of an animal it is protected against anaphylactic shock, the antigen being bound by these free antibodies so that it cannot enter the cells (anti-anaphylaxis). Evidently, then, the union of antigen and antibody in the circulating blood and in the presence of complement does not lead to the formation of sufficient poison to produce anaphylactic shock, whereas minute amounts of antigen entering a sensitized tissue produce prompt reactions. Similar results are obtained when the experiments are carried out *in vitro* under much more quantitative conditions.[52]

6. Antigens produce their specific effect on sensitized muscles in the absence of blood, whereas the presence of blood containing an abundance of free antibodies prevents the anaphylactic effect, these conditions both being exactly opposite to what would be the case if anaphylactic shock resulted from the production of a poison or anaphylatoxin by the action of circulating antibody and complement on the antigen.

7. A sensitized animal may be made highly tolerant to anaphylatoxin by repeated injection without at the same time losing its sensitivity to the specific antigen. On the other hand, its desensitization by injection of the specific antigen does not render it refractory to anaphylatoxin. Therefore, it would seem that poisoning by anaphylatoxin cannot be responsible for the reaction that takes place when antigen comes in contact with sensitized tissues (Dale and Kellaway).[52]

8. In the recorded anaphylatoxin experiments the existence of capillary embolism or endothelial intoxication has not ordinarily been excluded, and there is reason to believe that most of the observed symptoms are anaphylactoid from pulmonary embolism rather than anaphylactic.

9. Anaphylatoxins produce symptoms which are not identical with those of true anaphylactic shock, and they do not act on isolated uterine muscle, but only exhibit their action in the presence of circulating blood, apparently through the formation of complexes which keep the colloids finely dispersed.[52]

10. The effect of anaphylatoxins differs from that of true anaphylaxis in that the former produce much more decrease in the number of blood platelets and much more alteration in the capillary endothelium as manifested by edema (Dale and Kellaway).

11. All attempts to demonstrate that the blood of animals in anaphylactic shock contains a poison responsible for the observed manifestation have failed. (See Weil.) [53]

12. The effect of anaphylatoxin on the pH and surface tension of the blood is not the same as that seen in anaphylactic shock.[54]

13. The theory of the dependence of anaphylactic intoxication upon the formation of non-specific poisons from proteins by some sort of ferment reaction, received its most severe blow in the clear-cut experiments of Dale [55] with the guinea pig uterus. If we grant, as it seems we must, that stimulation of non-striated muscle is an essential, and probably the essential feature of acute anaphylactic shock, the Schultz-Dale method of studying anaphylaxis by recording the contraction of intestine or uterus strip from a sensitized animal when brought in contact with the specific antigen, portrays the reaction without the possible invalidating effects of capillary thrombosis or agglutination emboli. The fact that, as Dale points out, the specific antigen, when introduced into a bath containing the muscle strip, produces a reaction *immediately it comes in contact with the muscle,* and quite as promptly as a preformed, diffusible stimulant, such as histamine, pilocarpine or pituitary extract, is difficult to reconcile with the theory that the stimulating agent is liberated through proteolysis. Moreover, says Dale, "Apart from the absence of a coarse latent period, the rest of the time relations of the effect are very difficult to harmonize with any form of ferment theory. On such a theory, one would expect a gradual onset and slow rise to a maximum. But the onset is sudden, and the rate of progress to the maximum, when sensitiveness is fully developed and the dosage not too minute, is apparently limited only by the contraction rate of the plain muscle. After a few minutes at the maximum the effect begins to decline, and the muscle is then insensitive to further doses. In the form of the contraction curve, as in the brief latency, the effect recalls that of a powerful stimulant drug added directly to the bath."

These numerous observations cited above seem to exclude not only the humoral site of the anaphylaxis reaction, but also to rule out the possibility of its dependence on even an intracellular digestion of a sensitized antigen by a ferment, whether serum complement or intracellular proteolytic enzymes.

[53] *J. Immunol.,* 2:399 (1917).
[54] Zunz and La Barre, *Compt. rend. soc. biol.,* 89:676 (1923).
[55] *J. Pharm. Exp. Therap.,* 4:167 (1913).

Relation of Anaphylaxis to Histamine

But we cannot escape the fact that the manifestations of anaphylactic shock resemble in all respects those of an acute intoxication; furthermore, that histamine, the substance which produces the picture most closely resembling that of typical anaphylaxis, is a product of protein cleavage. (See Abel and Kubota;[56] Dale;[57] Hanke and Koessler.[58]) Not only does histamine cause bronchial spasm in guinea pigs, obstruction to pulmonary circulation in rabbits and fall of blood pressure in dogs, but applied to the skin or mucous membranes, it causes marked local urticaria resembling closely that of skin reactions in sensitized persons, and it does all these things in extremely minute dosage, comparable with the dosage of proteins used in anaphylactic reactions. Furthermore, its antecedent amino acid, histidine, is present in every known complete protein. Some other pure chemical products of protein cleavage, such as methyl guanidine, have more or less similar physiological effects. Manwaring [59] has observed evidence that some of the manifestations of anaphylaxis in dogs depend on substances liberated from the liver which have a histamine-like effect, although probably not histamine itself but an antigenic protein.

The chief respects in which histamine fails to account for all the phenomena of anaphylaxis are:

1. It fails to desensitize sensitized animals or tissues, yet produces strong reactions in the uterus strip that has been thoroughly desensitized (Dale).[55]

2. Histamine does not produce the temperature reactions usual in anaphylaxis.

3. Histamine does not produce the changes in coagulability of the blood usual in anaphylaxis.

4. Quinine augments the susceptibility of sensitized animals to the foreign protein but does not affect the intoxication produced by histamine (M. I. Smith).[60]

In respect to item one of the above list, we should not expect histamine to desensitize if it is the active product of an antigen-antibody reaction, since it is this reaction alone that is prevented by desensitization. As to items two and three, it may well be that these phenomena result from other products of the antigen-antibody reaction, since pre-

[56] *J. Pharm. Exp. Therap.*, **13**:243 (1919).
[57] *Johns Hopkins Hosp. Bull.* **31**:257, 310 (1920).
[58] *J. Biol. Chem.*, **43**:521-579 (1920).
[59] Manwaring *et al.*, *J. Immunol.*, **20**:567 (1925); **13**:63 (1927).
[60] *J. Immunol.*, **5**:239 (1920).

sumably many different substances are produced. The effect of quinine (item four) might be found to be an influence on the antigen-antibody reaction if this possibility were investigated. And at least histamine seems to have a synergetic relation to anaphylactic shock, probably because the points of attack of histamine and anaphylactic reactions are identical.[60]

The Significance of Anaphylatoxin Formation

It being established that when immune sera act on specific antigens, proteolysis occurs, and since the intermediate products of proteolysis are unquestionably toxic, how can we discard entirely the possibility that such substances do arise, and play at least some part in anaphylaxis, even if we can exclude proteolysis as the responsible factor in the immediate contraction of non-striated muscle both *in vivo* and *in vitro*? Furthermore, since the so-called anaphylatoxins are so readily produced, and exert such a profound physiological effect, their importance in human pathology must be considerable, even if they are not responsible for typical acute anaphylactic shock. Such observations as those of Quenu and Delbet,[61] Cannon and others,[62] on the responsibility of disintegration of traumatized muscle tissue for traumatic shock, come to mind in this connection.

Moreover, in the so-called Abderhalden reaction we have evidence that protein cleavage does occur in mixtures of immune serum and specific protein, and that in this process highly active toxic substances are produced (Bronfenbrenner).[63] For whatever may be said concerning the specificity of this reaction, there undoubtedly does commonly occur a greater amount of proteolysis with the specific antigen than if some other protein is present, and Manwaring [64] has obtained evidence that foreign proteins injected into animals sensitized to these same proteins lose their original character much more quickly than in unsensitized animals or in thoroughly immunized animals. Nor can we afford to overlook the important fact that racemized proteins (see p. 46), which are characterized by being incapable of attack by enzymes *in vitro,* or of being digested and metabolized *in vivo,* are also incapable of serving as antigens and producing anaphylactic intoxication, although derived from proteins which in the original state are highly antigenic.

Although we are justified in defining sharply the limits of what shall

[61] Delbet, *Rev. Chir.*, 57:309 (1919).
[62] Cannon, *Compt. rend. soc. biol.*, 81:850 (1918).
[63] *J. Exp. Med.*, 21:480 (1915).
[64] Manwaring *et al.*, *J. Immunol.*, 13:357 (1927); 15:109 and 351 (1928).

be considered as true anaphylaxis in discussing and studying that topic, related phenomena of allergy, such as anaphylatoxin formation, which we must exclude as outside those limits, are not therefore any less important. They merely are other problems, and presumably related problems, but how much bearing they have on specific anaphylaxis, if any, we do not know. From this open viewpoint the extensive studies of Novy [65] and his collaborators are of interest as a contribution to our knowledge of serum toxicity. They corroborate and extend the findings of others,[66] that the serum of a guinea pig may be made toxic for guinea pigs after incubating with agar, inulin, or small formed particles, such as trypanosomes or inorganic precipitates, so that from 1 to 3 cc. may be a lethal intravenous dose. Rat serum, which is toxic to guinea pigs even when untreated (3 to 4 cc. sometimes being fatal) may have its toxicity enhanced until 0.25 cc. is a lethal dose. Also, agar and other substances which render serum poisonous *in vitro* are very toxic when injected intravenously, as little as 9 mgm. of agar per kilo of guinea pig being fatal. Even unformed substances may render serum toxic (e.g., peptone solutions), and distilled water is said to produce "anaphylatoxin" in rat serum, which Sachs [67, 68] would explain by the precipitation of the serum globulins by the distilled water, thus disrupting the colloidal equilibrium and permitting the formation of toxic forms of colloids. But the proof that such phenomena are the same as those underlying the specific antigen-antibody reaction of anaphylaxis has not been furnished, and the term anaphylatoxin is misleading, especially as we have as yet no proof that a toxic material is formed in and responsible for true anaphylaxis reactions. Moreover, the "anaphylatoxins" thus formed (better called "serotoxins") seem to have little effect on rabbits, although these animals are far from insusceptible to anaphylaxis. Nor can we reconcile with the known facts of anaphylaxis the observation that the mere coagulation of blood renders the serum highly toxic. Unquestionably the readiness with which blood becomes poisonous is an important phenomenon, and presumably it plays an important part in pathology, but that this phenomenon is responsible for, or even related to, anaphylaxis, has yet to be demonstrated.

Certain French investigators particularly have attempted to explain the manifestations of anaphylaxis as due to a disturbance in the colloidal equilibrium, with partial flocculation of the plasma colloids and possibly

[65] Novy and DeKruif, *J. Am. Med. Assoc.*, **68**:1524 (1917); this being a summary of ten articles in the *J. Infectious Diseases*, **20**:499-854 (1917).
[66] *See* review by Bordet, *Bull. Johns Hopkins Hosp.*, **32**:269 (1921).
[67] *Kolloid-Z.*, **24**:113 (1919).
[68] *Arch. Hyg.*, **89**:322 (1920).

of those of the tissues themselves. They refer to this supposed change as "colloidoclasie," and have developed numerous explanations of the phenomena of allergy and anaphylaxis as well as many proposed methods of treatment based upon this hypothesis. These views can scarcely be considered, for they are based on work in which no careful distinction is made between anaphylactoid manifestations and true anaphylaxis, between specific desensitization and non-specific reduction of irritability, between anaphylatoxin effects and specific antigen-antibody reactions; moreover, capillary thrombosis and pulmonary embolism have not been ruled out or even taken into consideration (e.g., according to Lumière,[69] there is no difference between the effect of injection of suspensions of $BaSO_4$ and true anaphylactic shock), and so on. None of the work with muscle preparations *in vitro*, which rule out the factor of capillary occlusion, seems to have been taken into account.

The Basis of Anaphylactic Shock

But if we discard the anaphylatoxin theory of anaphylaxis, we are left without an explanation of the very striking phenomena of anaphylactic shock, for no satisfactory substitute hypothesis has been proposed. Dale[55] suggested that "A disturbance of the conditions of colloidal solution is set up in the muscle fibre," which is not altogether satisfying, although it may well be true. It has also been suggested that the colloidal changes may be the result of precipitation occurring within the cell through the reaction between the antibody, assumed to be a precipitin, and the antigen which has diffused into the cell. Weil speaks of a "cellular discharge" which is even more vague than Dale's "colloidal disturbance."

Danysz[70] has proposed the hypothesis that anaphylaxis is merely an intracellular or intravascular "indigestion," because of the impossibility of transforming colloidal antigen into crystalloids, but this does not fit at all the known facts of the character of the reaction in isolated tissues. None the less, the fact that slight disturbances in the equilibrium of plasma colloids render them highly toxic is an outstanding fact, and it becomes easily understandable that a similar alteration in colloidal equilibrium within the cell protoplasm may produce equally profound intoxication of the cell, so that at present there is a growing tendency to seek an explanation of anaphylaxis in the domain of colloidal chemistry. (See Zinsser,[71] and Sachs.[67])

[69] *Compt. rend. acad. sci.*, 172:544 (1921).
[70] *J. Infectious Diseases*, 22:427 (1918).
[71] Zinsser, *Arch. Int. Med.*, 16:223 (1915).

Pathological Physiology

As stated previously, the effects characteristic of anaphylactic intoxication are seen to be, for the most part, the results of contraction of non-striated muscle, and apparently the characteristic features of acute anaphylactic shock in different species depend merely on differences in the distribution of non-striated muscle in the different species. We have abundant experimental evidence that unstriated muscle of many tissues or organs is stimulated to contraction in the anaphylactic reaction, which explains the micturition and defecation, and perhaps the erection of the hairs and exophthalmos observed in anaphylactic shock.

There is no known pathological anatomy of anaphylactic shock that cannot apparently be explained by such a mechanism as that described above. The anaphylactic poison, if there is one, would seem to be after the order of the alkaloidal poisons, since it produces immediate but transitory effects, without evident structural alterations in the tissues. The anatomical alterations that are observed, such as hemorrhages or waxy degeneration of the voluntary muscles of respiration,[72] are ascribable to the asphyxia and circulatory obstruction.[73] Longcope [74] and Boughton [75] have observed lesions in the kidneys, hearts and livers of rabbits and guinea pigs subjected to repeated anaphylactic shock, as well as to repeated protein injections without shock, but in view of the frequency of spontaneous lesions in animals kept under similar conditions and subjected to much manipulation, it cannot be said positively that the lesions described resulted either from anaphylactic shock or from foreign proteins independent of the anaphylaxis. (See Bell and Hartzell.)[76] Nevertheless, we have in the Arthus phenomenon [77] a striking proof that local tissue injury may result from anaphylactic reactions, whether through direct cellular injury or protracted vascular occlusion, and so it would seem eminently probable that tissue injury may result elsewhere than in the skin under similar conditions.

Manwaring [78] has found that if the lungs of a dog sensitized to horse serum are perfused with a saline solution containing horse serum there

[72] Wells, *Centr. allgem. Pathol.*, **23**:945 (1912).

[73] The resistance of the red cells to hemolysis is decreased by the acidosis of anaphylactic shock, and the serum may exhibit some autohemolytic activity. [Capiau, *Arch. intern. physiol.*, **25**:167 (1925). *See also* Mendeleeff *et al.*, *ibid.*, **24**:145 (1925).]

[74] *J. Exp. Med.*, **18**:678 (1913); **22**:793 (1915).

[75] *J. Immunol.*, **4**:213 (1919).

[76] *J. Infectious Diseases*, **24**:618 (1919).

[77] The Arthus phenomenon consists in a local reaction, observed best in rabbits, which after sensitizing with one or several doses of an antigen, receive an injection of this antigen into the subcutaneous tissues. This is followed by a severe local inflammatory reaction, often with necrosis and sloughing of the skin. [*See* Opie, *J. Immunol.*, **9**:231 (1924).]

[78] Manwaring *et al.*, *J. Am. Med. Assoc.*, **80**:303 (1923).

occurs a tremendous pulmonary edema, and he believes this increased permeability of the capillaries to be the dominant fundamental change in anaphylaxis reactions.[79] He has attributed the effects of anaphylaxis to some substance liberated from the liver, exhibiting a histamine-like action on the vessels, for such reactions are not observed in sensitized dogs after removal of the liver, but will appear in a normal dog into which the liver of a sensitized dog has been transplanted.[80]

Presumably the decreased coagulability of the blood observed in anaphylactic shock depends on injury to the liver cells and release of anti-coagulating substances, for Weil found that blood containing the specific antigen loses its coagulability when perfused through the liver of a sensitized dog, and poisons which injure the liver produce a similar decrease in coagulability. Also, autolysis of the liver is said to be hastened in sensitized animals.[81] There seems to be no alteration in the epinephrine content of the adrenals in anaphylaxis.[82]

Apparently some change takes place in antigens which enter the anaphylactic liver, for Manwaring and Crowe [83] found that antigen perfused through the liver of a sensitized guinea pig lost its toxicity for sensitized guinea pig lungs, although normal livers did not have this effect, and Falls [84] observed that larger doses of antigen are required to produce shock when injected into the portal vein than when introduced into the jugular. Weil [85] has found evidence that antibody and antigen coexist within the reacting tissues for some time, the antigen being gradually destroyed, a process which may perhaps be accompanied by injury to the cells.

Although we recognize the systemic anaphylactic reaction only as it affects non-striated muscle tissue, it may well be that this is merely because such tissue alone registers the effects in a graphic manner. We do not know whether any or all other cells of the sensitized animal are affected when they come in contact with the specific antigen,[86] although there is reason to believe that this is the case.[87] Still less do we know what happens within the sensitized muscle cell when the antigen and antibody meet. Dale has shown that the response is immediate, as

[79] Questioned by Drinker and Bronfenbrenner [*J. Immunol.*, **9**:387 (1924)].
[80] Manwaring *et al.*, *J. Immunol.*, **10**:575 (1925).
[81] Hashinoto and Pick, *Arch. exp. Path. Pharm.*, **76**:89 (1914); Fenyvessy and Freund, *Biochem. Z.*, **96**:223 (1919).
[82] Smith and Ravitz, *J. Exp. Med.*, **32**:595 (1920).
[83] *J. Immunol.*, **2**:517 (1917).
[84] *J. Infectious Diseases*, **22**:83 (1918).
[85] *J. Immunol.*, **2**:399 (1917).
[86] No evidence of anaphylactic effects could be obtained with cells in tissue cultures by Meyer and Loewenthal [*Z. Immunitäts.*, **54**:420 (1928)].
[87] Frölich, *Z. Immunitäts.*, **20**:476 (1914).

when a potent diffusible drug acts on a cell, but whether the reaction changes the colloidal state of the cell, as has been vaguely suggested, or whether it causes a discharge of energy after the order of an electrical stimulus, or something entirely different, are matters concerning which we have not the slightest information.

Chemical Changes in Anaphylaxis

Metabolism studies may show an increased toxicogenic destruction of protein,[88] but the increase in amino acids, presumably resulting from proteolysis within the tissues of the reacting individual, is not large enough, if it occurs at all, to be significant. Thus, Auer and Van Slyke[89] were unable to find a demonstrable increase in free amino nitrogen in the anaphylactic guinea pig lung, nor do the livers of guinea pigs dying in anaphylactic shock contain appreciably more non-coagulable nitrogen than normal livers.[90] However, in anaphylaxis in guinea pigs, as well as after peptone poisoning, there is a considerable increase in non-coagulable nitrogen, creatinine and urea in the blood,[91] as well as a slight increase in amino nitrogen, but it is not known whether this comes from the tissues or from the antigen-antibody reaction in the blood, although the former seems more probable.[92] Manwaring[93] found that no appreciable loss of the antigenic protein occurred during its perfusion through the organs of sensitized animals. The gas metabolism of the animal as a whole and its tissue respiration are decreased in true anaphylactic shock.[94] The asphyxia of anaphylaxis is associated with marked acidosis,[95] and when the carbon-dioxide combining capacity of the blood falls below 25 volume per cent the animal usually dies. With this acidosis is a demonstrable fall in the pH of the plasma,[96] a marked increase in lactic acid,[97] and a decrease in the surface tension.[98] Numerous other changes have been described in the blood of animals dying in anaphylactic shock,[99] including an increase in the total

[88] Major, *Deut. Arch. klin. Med.*, **116**:248 (1914).
[89] *J. Exp. Med.*, **18**:210 (1913).
[90] Barger and Dale, *Biochem. J.*, **8**:670 (1914).
[91] Major, *Johns Hopkins Hosp. Bull.*, **34**:104 (1923).
[92] *See* Hisanobu, *Am. J. Physiol.*, **50**:357 (1920).
[93] Manwaring, Kusama and Crowe, *J. Immunol.*, **2**:511 (1917).
[94] Abderhalden and Wertheimer, *Arch. ges. Physiol. (Pflüger's)* **195**:487 (1922).
[95] Eggstein, *J. Lab. Clin. Med.*, **6**:555 (1921); Hirsch and Williams, *J. Infectious Diseases*, **30**:259 (1922); Zeckwer and Goodell, *J. Exp. Med.*, **43**:57 (1925).
[96] However, according to Mendeleef [*Arch. intern. physiol.*, **21**:15 (1923)] the changes in pH in anaphylactic shock are not always downward.
[97] McCullough and O'Neill, *J. Infectious Diseases*, **37**:225 (1925).
[98] Zunz and La Barre, *Compt. rend. soc. biol.*, **88**:990 (1923); **90**:658 (1924); **92**:223 (1925); Ramsdell, *J. Exp. Med.*, **47**:993 (1928).
[99] Wittkower, *Klin. Wochschr.*, **2**:450 (1923).

proteins,[101] with alterations in their colloidal behavior so that they are more precipitable by various means; also marked reduction in the number of leucocytes and platelets and in the coagulability of the blood, together with some increase in the refractive power, but not in the rotatory power.[102] There has also been described an increase in the blood sugar,[95, 97] an increase in cholesterol,[103] and a slight and irregular decrease in sodium, potassium, magnesium and calcium.[104] In dogs the glycogen disappears from the liver.[105] Possibly the observed reduction in viscosity of the blood may be of significance.[106]

There is evidently a marked change in endothelial permeability in anaphylactic intoxication,[107] as shown by the local edema and an augmented flow of thoracic lymph containing an increased protein content, the globulin and fibrinogen being especially increased when the shock is severe.[108] Also the osmotic pressure of the peritoneal fluid is increased in guinea pigs after anaphylactic shock.[109] If the phagocytic endothelial cells of the body as a whole are occupied by previous injections of finely divided suspensions, such as oxide of iron [110] or india ink [111] the animal is protected from anaphylactic shock, indicating the importance of these cells in anaphylaxis as in other immunity reactions,[112] but as yet it is not clear how this occupation of the reticulo-endothelium by phagocyted particles modifies anaphylactic shock.

Desensitization and Anti-anaphylaxis [113]

An important and characteristic feature of the anaphylactic reaction is the refractory condition which the recovered animal exhibits immediately thereafter, provided that the amount of antigen injected has been sufficiently large, and isolated muscle strips from sensitized animals exhibit the same phenomenon of being refractory after having reacted to the antigen. If the reaction is produced by only minimal quantities of antigen, the animal may still react to a second dose of the same antigen, so evidently this condition of desensitization depends

[101] Wichels [*Z. ges. exp. Med.*, **53**:287 (1926)] states that allergic organisms react to non-specific injections by an increase in globulins, which normal organisms do not do.
[102] Abderhalden and Wertheimer, *Arch. ges. Physiol.* (*Pflüger's*) **197**:85 (1922).
[103] Zunz and La Barre, *Compt. rend. soc. biol.*, **93**:1044 (1925).
[104] Azzi, *Arch. sci. med., Torino*, **45**:356 (1922). Potassium is increased in the serum in shock, according to Schittenhelm *et al*, *Z. ges. exp. Med.*, **58**:662 (1928).
[105] O'Neill, Moy and Manwaring, *J. Immunol.*, **10**:583 (1925).
[106] Waud, *Am. J. Physiol.*, **81**:165 (1927).
[107] *See* Hanzlik and De Eds, *J. Pharm.*, **29**:485 (1926).
[108] Petersen and Levinson, *J. Immunol.*, **8**:323 (1923).
[109] Fleisher and Mayer, *J. Immunol.*, **9**:319 (1924); **12**:19 (1926).
[110] Petersen *et al.*, *J. Immunol.*, **8**:367 (1923).
[111] Moldovan and Zolog, *Compt. rend. soc. biol.*, **89**:1242 (1923).
[112] *See also* Ehmer and Hammerschmidt, *Klin. Wochschr.*, **7**:931 (1928).
[113] *See* review by Longcope, *Physiol. Rev.*, **3**:240 (1923).

upon an exhaustion or saturation of the antibodies. The desensitization is quite as specific as the acute reaction itself, for if the guinea pig has been sensitized to two different proteins and recovers from the shock induced by one of them to which it thus becomes entirely refractory, it will still react to the second protein.

Weil [114] made a quantitative study of desensitization following passive sensitization with standardized amounts of antibody, and found that the quantity of antigen needed to produce a reaction in a partially desensitized pig did not bear a constant relation to the amount of unsaturated antibody presumably left after the first reaction, as shown by the following table:

Sensitization: 0.3 cc. immune serum intraperitoneally

Guinea pig series	Desensitizing dose of horse serum cc.	Minimal anaphylactic dose of horse serum, intraperitoneally cc.
1	0.005	0.1
2	0.01	0.5
3	0.02	0.5
4	0.05	0.5

As the minimum anaphylactic dose of antigen after partial desensitization is much larger than before partial desensitization, it would seem that the reactivity or affinity of the antibody had been much reduced in the desensitizing process. This alteration in quantitative relationship between antigen and antibody is distinctly different from the fixed quantitative relations observed between precipitinogen and precipitin, and Weil pointed out the resemblance to the Danysz phenomenon observed in toxin-antitoxin neutralization; i.e., the changed quantitative relationship produced by adding antitoxin to toxin in small fractions instead of a single neutralizing quantity.

Anti-anaphylaxis

Sensitized animals may be refractory to anaphylactic reactions not only because of saturation or exhaustion of the fixed antibodies, but because the presence of sufficient excess of free antibodies in the circulating blood may prevent the antigen from coming in contact with the intracellular antibodies, i.e., the condition of *anti-anaphylaxis*. There has been much confusion in the literature because of the careless use of the term "anti-anaphylaxis" to cover all refractory conditions. This

[114] *J. Immunol.*, 2:469 (1917).

term should logically be applied only to a resistance due to antibodies, using the term *desensitization* for the condition which it describes, as indicated in previous paragraphs.

It is this form of antibody resistance, true specific anti-anaphylaxis, which accounts for the frequent failure to obtain anaphylactic shock in guinea pigs immunized to a foreign protein by repeated injections. This assumption, that the refractory condition depends on the presence of free antibodies in the circulating blood, which was made early in anaphylaxis work, seems to have been converted into a certainty by the demonstration of Manwaring and Kusama [115] that the isolated organs of such immunized anti-anaphylactic guinea pigs are, when washed free of circulating antibodies, still highly sensitized to the specific antigen. Weil [116] previously showed that if small doses of antigen are injected subcutaneously into passively sensitized guinea pigs that have been given intravenous injections of large protective doses of antiserum, the uterus is not desensitized as it otherwise would be, since the antigens are kept by the free antibodies in the serum from uniting with the intracellular antibodies. Similar antianaphylaxis may be observed with the sensitized uterus *in vitro* if there is an excess of antibody in the fluid bathing it.[117]

I have found that the condition of anti-anaphylaxis may be produced through protracted feeding with a protein as well as by injection,[118] and presumably this alimentary immunization explains a number of phenomena observed both in experimental animals and in man.[119]

Anti-sensitization

Anti-sensitization is a somewhat similar phenomenon described by Weil.[120] If a guinea pig is given a single dose of rabbit serum several days before a sensitizing dose of serum from a rabbit immune to a foreign protein, the usual passive sensitization to this protein does not take place. This is explained by the development in the guinea pig of antibodies to the rabbit serum, which protect the guinea pig's tissues from the antibodies of the immune rabbit serum. In proof of this conclusion is the fact that such preliminary injection with rabbit serum does not prevent passive sensitization with the serum of a guinea pig immunized to foreign protein.

Another form of refractiveness, which is essentially a desensitiza-

[115] *J. Immunol.*, **2**:157 (1917).
[116] *J. Med. Res.*, **30**:299 (1914).
[117] Dale and Kellaway, *J. Physiol.*, **54**:cxliii (1921).
[118] *J. Infectious Diseases*, **9**:147 (1911).
[119] *See* Ratner, *Am. J. Diseases Children*, **36**:277 (1928).
[120] *Z. Immunitäts.*, **20**:199 (1913).

tion, may occur when a large sensitizing dose is given. Differences may be observed even when the large sensitizing doses are not very large; thus, Thomsen [121] states that a sensitizing dose of 0.004 cc. of serum produces a maximum sensitization more quickly than 0.1 cc., although the final maximum degree of sensitization is the same with each dose. This seems explainable on the basis of the persistence of antigens in the blood during the time of formation of the antibodies and their fixation within the cells, whereby these antibodies are saturated with the antigen gradually as fixation takes place, without at any one time sufficient reaction taking place to be observable. Of course, when a very large sensitizing dose of antigen is given there may also be engendered sufficient antibodies to produce the condition of anti-anaphylaxis from the excess antibodies in the blood.

Concurrence of Antigens

Still a different form of interference with sensitization has been studied by Julian Lewis,[122] who found that an amount of protein that will produce a marked anaphylactic sensitization when injected alone into a guinea pig, will fail to do so if injected together with, or within twenty-four hours after, a much larger amount of another protein. Thus, in a series of guinea pigs sensitized with 0.1 cc. horse serum mixed with decreasing quantities of dog serum from 2 cc. to 0.001 cc., no reactions were obtained on injecting horse serum into the animals receiving from 1 to 2 cc. of dog serum, severe reactions but with recovery in those receiving from 0.25 to 0.01 cc. dog serum, as compared with promptly fatal reactions in the animals that received no dog serum.

The serum from a rabbit immune to horse serum, which will passively sensitize a guinea pig to horse serum, will fail to do so if injected with, or within twenty-four hours after, a large dose of another protein.

These results may be explained by the conception that the number of receptors in the body that can unite with a foreign protein is limited. The inhibiting protein, if present in large amount, combines with all, or almost all, of these receptors. Hence, another protein injected with it, or after it, in small amounts is prevented from being combined in sufficient amount to stimulate the active production of antibodies for this second protein. And when a large amount of protein is injected with or immediately after a sensitizing dose of immune serum, the

[121] *Z. Immunitäts.*, 26:213 (1917).
[122] *J. Infectious Diseases*, 17:241 (1915).

combination of the latter with the cell receptors, which is necessary for passive sensitization, is prevented in the same way.

Non-specific Transitory Reduction of Reactivity

The intensity of anaphylactic reactions may be reduced by anesthetics and narcotics,[123] and also by intravenous injection of numerous sorts of substances shortly before giving the intoxicating dose of antigen. Peptones, trypsin, various inorganic salts,[124] urine and foreign proteins other than those used in sensitizing, are among these non-specific desensitizers, and their mechanism of action is not understood.[125] Kopaczevski and Vahram[126] have found that sodium oleate has this effect to a marked degree, which they ascribe to a lowering of surface tension of the blood, on the hypothesis that anaphylactic shock depends upon a plugging of capillaries by colloidal flocculation. Although perfusion experiments demonstrate that such gross capillary occlusion does not occur in the anaphylactic lung, yet the various items of evidence that changes in the colloidal state of the blood are of importance in the phenomena of "anaphylatoxin" intoxication make this observation of significance. The protective action of NaCl has usually been ascribed to its interference with complement action, but Richet[127] attributes it to its action on nerve cells, and others would ascribe it to the effect on the colloidal state of the cells and fluids reducing their irritability. Thomsen[121] refers such non-specific interference with the anaphylaxis reaction to a reduction in the speed of reaction between antigen and antibody, whereby a greater amount of antigen is found necessary to produce shock, but no explanation is offered as to why the speed of reaction is reduced.

Many of the agents which produce non-specific desensitization undoubtedly do it by altering the sensitivity of the reactive mechanism. It has been learned that when sensitization is accomplished with two antigens simultaneously, reaction against one leaves the animal, or the uterine strip, still capable of reacting to the second antigen, but less vigorously than it did to the first. If the antigens used are two unrelated pure proteins it will be found that if a period of rest is allowed

[123] *See* Elkeles, *Z. Immunitäts.*, **54**:471 (1928).
[124] *See* Klopstock, *Klin. Wochschr.*, **4**:312 (1925).
[125] Kyes and Strauser [*J. Immunol.*, **12**:419 (1926)] reported inhibition by hirudin of anaphylactic shock in pigeons. Similar results were not obtained in guinea pigs by Loewenthal [*Z. Immunitäts.*, **54**:138 (1927)], Hyde [*Am. J. Hyg.*, **7**:614 (1927)] and others, but have been obtained by Van de Carr and Williams [*J. Immunol.*, **15**:13 (1928)] under certain conditions [*ibid.*, **15**:355 (1928)].
[126] *Compt. rend. acad. sci.*, **169**:250 (1919).
[127] Richet, Brodin and St. Girons, *Compt. rend. acad. sci.*, **169**:9 (1919).

for a few days before injecting the second antigen the reactivity is restored to the original degree, indicating that fatigue or some similar reduction in activity of response is responsible for the decreased reactivity immediately after the first reaction. Of course when complex antigens such as sera are used (as in the experiments of Karsner and Ecker) [128] group reactions by identical or related antigenic proteins undoubtedly come into play and more or less complete removal of the specific antibodies may then be responsible for the desensitization.

Recapitulation

The term allergy is used to cover all variations in reaction of living tissues to foreign chemical agents, whether antigenic or non-antigenic in character, or whether the change is towards hypersensitivity or reduced sensitivity. The term anaphylaxis is properly limited to the hypersensitiveness to antigenic substances which depends on a true antigen-antibody reaction. Any and all proteins which show antigenic capacity in inciting antibody formation, demonstrable by the other immunological reactions, are capable of producing anaphylactic sensitization and reaction. As yet it has not been shown that any non-protein substance is capable of producing anaphylactic sensitization, but it is possible that non-protein radicals may unite with the tissue or blood proteins of an animal and so alter them that they become as foreign proteins to that animal and thus incite anaphylactic reactions. Many substances which cause agglutination of red corpuscles or blood platelets, or alter the degree of dispersion of the blood colloids will, when injected intravenously, cause intoxication with symptoms in many respects like anaphylaxis, depending for the most part on occlusion of the pulmonary capillaries; these *anaphylactoid* manifestations are fundamentally different from true anaphylaxis reactions, despite the similarity in the symptoms.

The immune body responsible for the state of anaphylactic hypersensitivity is probably identical with the precipitins, and, in turn, with the complement-fixing antibodies, although the identity of these antibodies cannot be said to be fully established. There is good reason to believe that the anaphylactic reaction takes place within cells in which the antibody has been fixed, although the participation of substances formed by union of antigen and antibody in the circulating blood may account for some of the phenomena seen in anaphylactic shock. What changes are produced in the cells to bring about the reaction are not

[128] Karsner and Ecker, *J. Infectious Diseases*, 30:333 (1922).

known, but the reaction occurs so rapidly that the participation of any enzymatic process seems to be excluded. As yet we have no convincing evidence that either digestive proteolysis or, indeed, the formation of any sort of poison such as hypothetical "anaphylatoxin," is responsible for anaphylactic shock. The poisonous character of serum resulting from slight alterations in the state of its colloidal constituents may be very marked, but this seems not to account for anaphylactic shock. Neither does the toxic action of histamine or similar definite poisons seem to be responsible, however much histamine effects resemble some of the features of anaphylactic intoxication. The final explanation of the character of the change which brings about anaphylactic intoxication is still lacking.

Most of the manifestations of the reaction seem to be the result of marked contraction of non-striated muscle throughout the body, the differences in the character of the reaction in different species probably depending largely on differences in the distribution of non-striated muscle in the several species. Possibly other cells are also affected, although there is less evidence for this. That there is a profound upset of the body in anaphylactic shock is shown by the decreased coagulability of the blood, the rise in non-protein nitrogen in the blood, the marked acidosis and reduced gaseous metabolism of the animal as a whole and of its isolated tissues.

Animals may become refractory to anaphylactic reactions in several ways, namely, desensitization, through exhaustion of the fixed intracellular antibodies by their union with the antigen; anti-anaphylaxis, when there are sufficient free antibodies in the circulating blood to unite with all the antigen so that it cannot reach the sensitized tissues in which the reaction takes place; anti-sensitization against passive anaphylaxis, when the blood contains antibodies against the serum which contains the anaphylactic sensitizer, since the sensitizing antibodies cannot then reach the tissue cells; tissue inactivation, when through exhaustion, drug action or other injury, the sensitized cells cannot respond to the antigen-antibody reaction.

CHAPTER X

PHAGOCYTIC IMMUNITY

In the preceding chapters we have dealt chiefly with immunological reactions which take place without direct participation of the body cells, but in the defense of the body against infection the cells play an important part, and with many types of infection the cellular reaction seems to be the chief element in the defense.[1] The cellular defense is accomplished in part in a mechanical manner, the cells crowding about the infected area, walling in the bacteria so that their action is localized, as seen in the formation of abscesses and tubercles. In addition to this, however, the cells play a more active part, often taking the bacteria into their substance and destroying them. Chemical processes come into play in both of these defense reactions.

Chemotaxis

The accumulation of leucocytes at a given point in the body indicates that some means of communication must exist between this point and the leucocytes in the circulating blood. No direct communication by the nervous system or other structural method existing, the only possible explanation is that the communication is through the fluids of the body, and depends upon changes in their chemical composition or physical condition. As the latter generally depends upon the former, the communication is considered to be accomplished by chemical agencies, and called *chemotaxis*.

This process resembles closely the behavior of living organisms suspended in fluids, which has been studied extensively by biologists and found to obey definite laws. Leucocytes are much like the amebæ, not only in structure but even more in their behavior in response to stimuli, and in general seem to react much as does any other droplet suspended in a fluid medium when acted upon by substances which alter the surface tension. If the substance lowers the surface tension on one side of the droplet or cell, the surface will bulge on that side, the contents will flow into the new space so offered, and the rest of the wall will

[1] *See* review by F. P. Gay, *Physiol. Rev.*, 4:191 (1924).

contract; hence the drop moves toward the point of lowered surface tension. Conversely, if the tension is increased in one place, the wall at this point will contract with greater force than elsewhere, driving the contents toward the less resistant part of the surface, and the drop will move away from the point of increased tension.

That leucocytes come to the site of an infection because of chemical substances produced by bacteria at this point, that is to say, through chemotaxis, was first clearly pointed out by Leber, who likened the attraction of such substances for leucocytes to the effect of malic acid in attracting spermatozoids in certain plants. He found that in inflammation of the cornea, leucocytes invade the avascular cornea from the distant vessels, not in an irregular manner, but all move directly toward the point of infection. Since Leber's experiments, many other investigations have been made showing that chemical substances of many different origins other than bacterial, exert a chemotactic influence on leucocytes. Some substances are indifferent in effect, most are positive, while some are believed to repel leucocytes; i.e., are negatively chemotactic.

On the assumption that leucocytes obey the same laws in their motions as do the amebæ, studies of the latter and of other forms of protozoa have furnished most of the ideas, hypotheses, and theories of the forces involved in leucocytic activities. The structural relation of the leucocyte to the ameba is striking, although by no means complete; the relation of their activities is even closer. Each is a microscopic, independent, unicellular organism, moving freely in all directions by means of pseudopodia and protoplasmic streaming, taking other smaller bodies into its substance and digesting them, reacting similarly to like stimuli, and containing similarly a nucleus and many granules. That the unicellular protozoa, devoid of any central nervous system, and without any apparent co-ordinating mechanism, seem able to move about in a purposeful way, going toward food supplies and away from injurious agencies, toward or away from light, heat, and chemicals, has long attracted the interest of physiologists, particularly as in these single-celled organisms we may look for the simplest conditions of existence and the most elementary life processes.

Experiments have shown that many of the processes characteristic of amebæ and leucocytes, especially chemotaxis, ameboid motion and phagocytosis, may be imitated by oil droplets or similar suspensions made to react to substances which alter their surface tension.[2] Even

[2] *See* Rhumbler, Abderhalden's "Handbuch Arbeitsmethoden," 1921, Abt. v, Teil 3, Heft 2. Urban and Schwarzenburg, Berlin.

such complicated processes as the building of shells and cell division may be imitated closely by such "artificial amœbæ." These experimental observations I have discussed more in detail elsewhere.[3]

Such experiments indicate strongly, to say the least, that amebæ and presumably leucocytes, react to stimuli of various kinds, chiefly through the effect of these stimuli upon surface tension. The stimuli may come from within the cell, being in this case the result of changes in composition brought about by metabolic processes; such chemical products alter the tension of the surface nearest their point of origin, causing what appears to be spontaneous motion. Stimuli acting from without may be chemical, thermal, electrical, or mechanical, but in any event they appear to act as stimuli to motion through their effect upon surface tension; if they decrease the surface tension the cell goes toward them; if they increase the tension, the cell moves away.[4] The behavior of leucocytes in inflammation I have attempted to explain on these purely physical grounds,[5] as follows:

At the point of cell injury or of infection, substances are produced that exert positive chemotaxis, as can be shown by experiments both outside and inside the body; these substances are chemotactic because they influence the surface tension of the leucocytes, and since with most of the products of cell disintegration the effect is to lower surface tension, the chemotactic effect is positive. As the chemotactic substances are produced, they diffuse through the tissues until they reach the walls of a capillary, through which they begin to pass, presumably most rapidly through the thinnest parts of the wall, the "stomata" and intercellular substance. The leucocytes passing along in the bore of the capillary will be touched by the chemotactic substances most on the side from which the substances diffuse; the surface tension will be lowered on this side, causing the formation of pseudopodia and motion in this direction. When the leucocytes come in contact with the wall, their surfaces, because saturated with the chemotactic substances, will have a tension much the same as that of the cells of the capillary wall, which are likewise saturated with the same substances, and the two surfaces will tend to cling to one another, explaining the phenomenon of adhesion of leucocytes to the capillary wall, when, according to the usual description, "the leucocytes behave as if either they or the capillary

[3] Wells, "Chemical Pathology," 5th Edition, W. B. Saunders Co., Phila., 1925, Chapter XIII, pp. 286-294.

[4] For example, sodium salts of the lower fatty acids stimulate formation of pseudopodia in polymorphonuclear leucocytes by lowering surface tension [Bakker, *Arch. neerland. physiol.*, 11:234 (1926)].

[5] Wells, H. G., "Chemical Pathology," 1st Edition, W. B. Saunders Co., Philadelphia, 1907.

wall had become sticky." Surface tension of the leucocytes will be least nearest the points where the most chemotactic substances are entering the capillary; hence the pseudopodia will form in this direction and flow through the capillary wall, the rest of the cytoplasm flowing after and dragging the nucleus along in an apparently passive manner. Since it is the cytoplasm that seems to be chiefly affected in these processes, the nucleus appearing to be rendered inert by its relatively dense and fixed structure, the leucocytes with most cytoplasm are most active in migration, while those with the least, the lymphocytes, are affected relatively little or not at all. Once through the vessel wall, the motion continues in the same manner, toward the side from which the chemotactic matter comes, just as a mercury drop flows toward a crystal of potassium dichromate, or a drop of oil flows toward alcohol. If the leucocyte meets a substance that lowers its surface tension sufficiently, it will flow around the object and enclose it, just as a chloroform drop flows about a piece of shellac or balsam; this constitutes phagocytosis.

General leucocytosis can be explained equally well on the same grounds. Chemotactic substances from the area of inflammation enter the blood-stream, and so, in a very dilute form, pass through the bone-marrow. The chemotaxis of the blood will be greater than that of the marrow, and the leucocytes will move toward and into the blood. As long as the blood contains more chemotactic substances than the marrow, leucocytosis will increase, to stop when the amount in blood and marrow is alike or when there is less in the blood than in the marrow. The free cells of the tissues involved in inflammation can, of course, obey the same influences as the leucocytes, and apparently do so in so far as they are not checked by structural impediments to flowing motion. An illustration of the chemotaxis of epithelial cells is furnished by B. Fischer,[6] who found that stained fats cause growth and migration of epithelial cells in the direction of the fat.

Cells with much cytoplasm are best fitted to move freely, as a rule, and hence we see chiefly the large mononuclear cells of the lymph sinuses and the serous cavities, and the large hyaline and granular cells of the blood acting as phagocytes, for phagocytosis is apparently no different from ameboid motion which continues about a particle until it is surrounded. Likewise we see the "epithelioid" and large mononuclear cells with their abundant cytoplasm fusing together to form giant-cells. The method of growing tissue *in vitro* permits of observation of the process of giant-cell formation, and establishes that, for foreign body

[6] *Münch. med. Wochschr.*, **53**:2041 (1906).

giant-cells at least, they are formed by fusion of wandering cells (Lambert).[7] The formation of giant-cells is, on this ground, but an amplification of ameboid movement and phagocytosis. The fusing of the individual cells is due to the lowering of their surface tension by the materials diffusing from the body which is to be absorbed, until the surfaces of adjacent cells become alike, when the surface tension at the point where each cell is in contact becomes zero and the cytoplasm runs together.

Physical explanations of ameboid movement seem to fit well the known facts concerning the actions of leucocytes.[8] There arise but a few difficulties in applying these laws to leucocytic action; one is the phagocytosis of chemically inert bodies, such as coal particles, tattooing materials, stone dust, etc. We know that amebæ also may take up such inert materials, although they generally refuse them, and it is believed that the particles exert some local injury to the cell wall that leads to an alteration in its tension. Amebæ seem also sometimes to excrete a sticky substance over their surfaces or over the foreign matter that is to be engulfed, which excretion appears to be the result of surface stimulation. Possibly leucocytes do the same, or the proteins of the blood and tissues may coat the foreign particles, for Fenn[9] found that particles of carbon and quartz will not be taken up by leucocytes *in vitro* unless they are coated by a protective colloid.

We must bear in mind that the protoplasmic cells have much greater possibilities for action than the "artificial ameba," since within the protoplasm countless chemical changes are going on which must cause continual alteration in surface tension; it is quite possible that mere mechanical action may alter chemical action at the point of contact, so that the injuring particle may become surrounded through local liquefaction of the protoplasm. At the very least *the surface tension explanation of leucocytic action agrees perfectly with most of the observed actions of leucocytes,* and it seems to be the most reasonable theory offered. There appears to be no middle ground between some such physical theory and a metaphysical theory which would endow a single cell, without organs or nervous system, with the reasoning powers of highly developed animals, a position incompatible with the entire evidence of experience.

[7] *Anat. Record,* 6:91 (1912).
[8] *See also* Herrera, *Atti accad. Lincei,* 4:247 (1926); 5:245, 379.
[9] *J. Gen. Physiol.,* 3:465 (1921); 5:311 (1923).

A series of studies by Fenn [10] on the phagocytosis of simple inert particles, such as carbon and quartz, by rat leucocytes, led to results that seem to him not to be in harmony with the theory that phagocytosis is determined by surface tension alone, although they do not offer any other explanation. For example, he found that smaller particles were ingested less rapidly than particles of a certain optimum size, although from the principles of surface tension the reverse should be the case. Furthermore, it was found that leucocytes ingest quartz particles more rapidly than carbon particles in acid solutions, whereas the carbon is taken up better in faintly alkaline solutions, but in the presence of acacia the carbon is taken up better than quartz even in acid solutions, although theoretically in the presence of such a protective colloid as acacia the surface charges of different suspended particles should be the same as that of the protective colloid. Therefore, it becomes difficult to explain the differences in behavior of carbon and quartz particles in phagocytosis on the basis of endosmotic charges.[11] It is evident that the complete explanation of chemotaxis and phagocytosis still awaits further investigation, although it is safe to predict that the explanation will be of a physico-chemical nature in view of the evident close relation of the processes of chemotaxis and phagocytosis to surface tension phenomena.

The hypothesis that chemotaxis depends on osmotic forces alone has been advanced by Maltaner and Hoppe,[12] who found that leucocytes migrate into capillary tubes containing various substances only when these are in solutions of greater density than the leucocyte suspension. Since in doing this the leucocytes move in a direction with the water current and opposite to the diffusing substances, the deduction is made that their motion depends on osmotic forces; the leucocyte being relatively impermeable to the crystalloids in solution and relatively rich in water it moves with the water and against the crystalloids. In support of this they cite experiments with artificial leucocytes made by filling a small celloidin sac with water; such a sac moves towards strong solutions of crystalloids diffusing into water. Also, they found that if a piece of agar jelly containing 3% NaCl, or a casein-trypsin digestion mixture, is suspended in an emulsion of leucocytes these remain in contact with the agar from which the crystalloids are diffusing, whereas

[10] W. O. Fenn, *J. Gen. Physiol.*, 3:439, 575 (1921); 4:331, 373 (1922); 5:143, 169 (1923). *Also* review in "The Newer Knowledge of Bacteriology and Immunology," Chapter LXV, Univ. of Chicago Press, 1928.

[11] Ponder [*J. Gen. Physiol.*, 9:827 (1926)] has discussed the possible influence of the electric charge of cell and particle on phagocytosis. See also *Protoplasma*, 3:611 (1928) for a discussion of the physical factors involved in phagocytosis.

[12] *J. Hyg.*, 19:309 (1920).

if the agar contains no crystalloids the leucocytes gravitate to the bottom of the tube. Furthermore, they were unable to confirm Buchner's experiments which he advanced as showing that bacterial proteins are positively chemotactic, nor did they find other reputedly positive substances to have this effect when in hypotonic solutions. But it would seem that if positive chemotaxis depends solely on osmotic pressure, any hypertonic solution should be positively chemotactic, which is certainly not the case, whereas some of the substances most actively chemotactic *in vivo,* e.g., aleuronat, turpentine, are active in solutions that are probably of relatively low osmotic pressure.

Studies in my laboratory by Miss E. P. Wolf [13] showed that positive chemotaxis is exerted *in vitro* by certain substances and not by others, entirely independent of the osmotic pressure of the solutions. For example, the calcium ion was the only inorganic ion found to be positively chemotactic *per se,* but when in the form of calcium citrate the negative chemotaxis of the citrate ion neutralizes the positive effect of the calcium. Phosphates are positively chemotactic, except when combined with the negatively chemotactic potassium ion. It was also found that substances which produce a very acute inflammation, such as cantharidin, or turpentine, are positively chemotactic by this method, but substances, such as mustard gas, which produce a marked necrotizing effect are found to be negatively chemotactic, or neutral, though physiologically they would appear to be positively chemotactic. All amino acids and amines are positively chemotactic to a certain extent. It seems that the longer the carbon chain, the greater the degree of chemotaxis, though this is not absolute.

These studies of Miss Wolf's were made in the attempt to learn what chemical, physical or physico-chemical properties a substance must have in order to incite inflammation, or that it may be unable to incite inflammation, in the hope that it might be found possible to predict from the known chemical properties of a substance its capacity to cause inflammation. It seemed, from these and subsequent studies,[14] that *a substance capable of inciting inflammation must be both fat-soluble and positively chemotactic,* but not all substances that are positively chemotactic *in vitro* will produce inflammation *in vivo.* No laws could be established to account for the presence or absence of chemotactic influence in a given substance, closely related substances being some-

[13] *J. Exp. Med.,* 34:375 (1921)
[14] *J. Exp. Med.,* 37:511 (1923).

times contradictory in chemotactic effect, while altogether dissimilar substances may have similar properties in respect to chemotaxis.

Abramson [15] has advanced the hypothesis that a factor in determining migration of leucocytes may lie in the electromotive forces at play in the tissues, the leucocytes being negatively charged, and migrating cataphoretically to the relatively positive injured tissues. Feringa [16] observed that widely varying substances injected into the abdominal cavity of animals led to the exudation of many leucocytes, independent of the nature of the injected substance. He found that the pH of the abdominal exudate invariably fell to 7.2 from the normal of 7.6, without any relation to the initial reaction of the injected fluid, and if by injection of alkalies this development of acidity was prevented, the leucocytic accumulation was prevented. This suggests that positive chemotaxis depends at least in part on the effect of H-ions, in which he is supported by Graff [17] and Jochims,[18] but some conditions known to be associated with relatively high H-ion concentrations in the tissues, such as violent muscular activity [19] or local asphyxia, do not lead to noticeable leucocytic accumulation.

Phagocytosis [20]

The engulfing of bacteria, cells, tissue products, etc., by leucocytes seems to be but an extension of the phenomenon of chemotaxis. When the substance toward which the leucocyte is drawn is small enough, the leucocyte simply continues its motion until it has flowed entirely about the particle. Later the particle becomes, as a rule, more or less altered within the cell, unless it is a perfectly insoluble substance, such as a bit of coal-dust. This action upon the engulfed object is undoubtedly due to the action of intracellular enzymes.[21]

Whether bacteria are digested in leucocytes by the same enzymes that digest the leucocytes themselves after they are killed (i.e., the autolytic ferments), or by some specialized enzyme, is not known.[22] The eventual excretion of the remains of the bacteria or other foreign bodies

[15] *J. Exp. Med.*, 46:987 (1927).
[16] K. J. Feringa, *Proc. Acad. Sci. Amsterdam*, 25:36 (1922). Dissertation Groningen, 1922, Abst. in *Ber. ges. Physiol. exp. Pharmakol.*, 14:93 (1922); *Arch. ges. Physiol.*, 203:672 (1924).
[17] *Münch. med. Wochschr.*, 50:1721 (1922).
[18] *Arch. ges. Physiol.*, 216:611 (1927).
[19] Wells, *J. Exp. Med.*, 11:1 (1909).
[20] *See* review by Metschnikoff, Kolle and Wassermann's "Handb. d. Path. Mikroorganismen," 11:655 (1913); *also* H. J. Hamburger, "Physikalisch-chemische Untersuchungen über Phagocyten," Wiesbaden, Bergmann, 1912.
[21] See Opie, *Jour. Exp. Med.*, 8:410 (1906).
[22] According to Pulicher [*Boll. soc. ital. biol. sper.*, 2:722 (1927)] intracellular digestion of phagocyted leucocytes in the cells of a peritoneal exudate in a guinea pig occurs at a pH of about 4.4, or even as low as 3.7.

by the phagocytes is ascribed by Rhumbler to changes in the composition of the particles through digestion, so that they have a greater surface affinity for the surrounding fluids than for the protoplasm of the cell.

Calcium and magnesium salts increase phagocytosis as well as leucocytic migration, according to Hamburger [23] and others,[24] although Otsubo [25] found that m/8 solution of $CaCl_2$ as well as a series of other salts diminished phagocytosis of streptococci, weaker solutions not increasing phagocytosis. Other authors have found that changes in osmotic pressure decrease these activities, as also does quinine even in dilutions of 0.001 per cent. Phagocytosis cannot take place in the absence of electrolytes, according to Sawtchenko.[26] Fat-soluble substances in general increase phagocytosis (Hamburger),[27] but cholesterol inhibits phagocytosis [28] (its effects being suppressed by lecithin),[29] acting apparently by virtue of its OH group. Agents facilitating oxidation favor phagocytosis (Arkin).[30] Wadsworth and Hoppe [31] found that cultures of many sorts of bacteria contain substances of unknown nature which inhibit phagocytosis, these substances being absorbed by the leucocytes from which they can be removed by washing, with restoration of the phagocytic activity; they are distinct from true toxins if such are present in the cultures, are thermostable and not neutralized by immune sera.

Maximum phagocytosis occurs at the normal body temperature of the animal furnishing the leucocytes (Madsen and Wulff).[32] These authors believed that the increase of phagocytosis at rising temperatures from 5° to body temperature follows the van't Hoff-Arrhenius law for speed of reactions. Fenn,[33] however, found that the phagocytosis of bacteria does not follow the law for a monomolecular reaction, possibly because of the toxic effect of the bacterial products upon the leucocytes, for with such non-toxic particles as quartz and carbon the rate of ingestion gave a constant K when calculated for the formula of a monomolecular reaction.

Phagocytosis cannot be so readily ascribed to chemotaxis, however,

[23] *Biochem. Z.*, 26:66 (1910).
[24] Eggers, *J. Infectious Diseases*, 6:662 (1909); Nagai and Ito, *Mitt. Med. Fac. Univ. Tokio*, 25:25 (1920).
[25] *J. Infectious Diseases*, 28:18 (1921)
[26] *Arch. sci. biol., St. Petersburg*, 16:161 (1911); 17:128 (1912).
[27] Hamburger and de Haan, *Arch. Anat. Physiol.*, 1913, Physiol. Abt., p. 77.
[28] Dewey and Nuzum, *J. Infectious Diseases*, 15:472 (1914).
[29] Stuber, *Biochem. Z.*, 51:211 (1913); 53:493 (1914).
[30] *J. Infectious Diseases*, 13:418 (1913).
[31] *J. Immunol.*, 6:399 (1921).
[32] *Ann. inst. Pasteur*, 33:437 (1919).
[33] *J. Gen. Physiol.*, 3:465 (1921); 4:331 (1922).

in the case of phagocytosis of perfectly insoluble, chemically inert particles, such as coal-dust. The leucocytes seem to take up foreign bodies without reference to their nutritive value, absorbing India-ink granules and bacteria impartially when they are injected together, and loading themselves so full of carmine granules that they cannot take up bacteria subsequently injected. It is possible that foreign bodies first become coated with a layer of altered protein which then leads to phagocytosis. The nature of mechanical stimulation of cells is explained by Osterhout [34] as a chemical reaction to rupture of semipermeable cellular surfaces, and there is evidence from plant cells supporting this hypothesis, but its applicability to animal cells apparently has not been investigated.

Not only leucocytes but tissue cells are capable of moving and performing phagocytosis when properly stimulated, and apparently all or nearly all fixed cells may act as phagocytes under some conditions. Their power of independent movement is much less than their phagocytic power. Reticulo-endothelial cells are particularly active in phagocytosis, as also are the new mesodermal cells produced in inflammation. Apparently they obey the same laws as the leucocytes, and not only take up bacteria, but also fragments of cells and tissues, red corpuscles, and even intact leucocytes and other cells.

Results of Phagocytosis

After phagocytosis has been accomplished, the fate of the engulfed object depends upon its nature. If digestible by the intracellular enzymes it is soon destroyed, but in the case of engulfed living cells, it seems probable that they must be first killed—they form no exception to the rule that living protoplasm cannot be digested. This brings forward the question of so much importance in the problems of immunity: Do living bacteria enter phagocytes, or are they first killed by extracellular agencies before they can be taken up? At the present time it seems to be positively established that leucocytes do take up bacteria which are still viable, and which may either multiply inside the leucocytes or may be destroyed by intracellular processes. On the other hand, leucocytes do not take up extremely virulent bacteria, and hence the question as to the relative importance of the defensive rôles played by the leucocytes and by the body fluids is still undetermined.

Phagocytosis of living virulent bacteria may not always be an unmixed benefit. Besides the obvious possibility of transporting the bacteria and spreading infection, we have also evidence that through

[34] *Proc. Natl. Acad. Sci.*, 2:237 (1916).

phagocytosis living bacteria may be protected against the action of bactericidal substances in the blood and tissues.[35]

Leucocytes contain substances which are strongly bactericidal, independent of the action of the blood serum, and which have been called *endolysins;*[36] they are resistant to temperature of 65° or even higher, and seem to be bound rather firmly to the protoplasm of the leucocytes, for they resist extraction except by vigorous methods; they have a complex structure like the amboceptor-complement bacteriolysins or alexins of the serum, and are not specific (Weil).[37] They do not pass through porcelain filters readily, are precipitated by saturation with ammonium sulfate, and resemble the enzymes in many respects.[38] It is probable that the endolysins act upon bacteria that have been phagocyted, and perhaps also upon free bacteria when liberated through disintegration of the leucocytes. Lymphocytes and macrophages seem to be devoid of this endolysin.[39]

Opsonins (Tropins)

The phagocytosis of bacteria is not merely a matter of reaction between the bacteria and the cells, however, for Wright and Douglas demonstrated that certain substances in the blood serum are necessary to prepare the bacteria for phagocytosis, these substances being termed by them *"opsonins."* If leucocytes are washed free from serum with salt solution and let stand in a test-tube with such bacteria as *Streptococcus hemolyticus, Staphylococcus pyogenes, B. typhosus, B. coli, B. tuberculosis,* and various other organisms, no phagocytosis occurs. If, however, some serum from a normal or an immunized animal is added to the mixture, active phagocytosis soon takes place. The action of opsonins is also involved in phagocytosis by endothelium.

The name *bacteriotropin* was introduced by Neufeld and Rimpau[40] for the same substances, found by them to be present in antistreptococcus and antipneumococcus serum and responsible for a stimulating effect on phagocytosis of these cocci.

The opsonins or tropins are definitely antibodies, being increased in the blood of animals immunized against bacteria and other cells, and showing the usual specificity of immune antibodies. There are also

[35] Rous and Jones, *J. Exp. Med.*, 23:601 (1916).
[36] For general review *see* Kling, *Z. Immunitäts.*, 7:1 (1910).
[37] *Arch. Hyg.*, 74:289 (1911).
[38] Manwaring, *J. Exp. Med.*, 16:250 (1912).
[39] Schneider, *Arch. Hyg.*, 70:40 (1909).
[40] *Deut. med. Wochschr.*, 30:1458 (1904).

"normal opsonins," present in the serum of unimmunized animals, just as with other "normal" antibodies.

Although there have been many expressions of the opinion that the opsonins are not distinct antibodies, but are identical with agglutinins,[41] bacteriolytic amboceptors, or other antibodies, there is evidence to the contrary.[42] The union of opsonin and bacteria seems to follow the same quantitative laws as other antigen-antibody reactions (Amato).[43]

There are two opsonizing elements in serum, one thermostable and one thermolabile, it being the former which is increased during immunization; the thermostable element unites firmly with the object which is to be opsonized, while the thermolabile element seems to remain free in the fluid (Hektoen), and is perhaps identical with complement.[44] Dean [45] calls attention to the fact that in tuberculosis the opsonic index for the tubercle bacillus is decreased, while the same serum shows an increased complement fixation reaction, a fact which speaks against the identity of the two antibodies involved, i.e., opsonins and amboceptors. However, it is possible to imagine that the low opsonic index in active tuberculosis may depend on the presence of inhibiting substances rather than on a decrease in opsonins.

Sawtchenko [46] holds that there are two steps in phagocytosis; (1) Fixation of the bacteria to the leucocyte because of modification of surface tension by the fixative substance (opsonin or amboceptor-complement complex); (2) Ameboid motion of the phagocyte; an entirely independent phenomenon. Neither phase of phagocytosis can occur in the absence of electrolytes.

It would seem that opsonization and phagocytosis constitute but one of a series of similar processes by which foreign proteins are removed from the blood and tissues; i.e., by lysis by extracellular enzymes when this is possible, as it is with simple protein aggregates (albuminolysis) and with some of the more labile cells (hemolysis, bacteriolysis); but in the case of more resistant structures, notably Gram-positive cocci and acid-fast bacilli, extracellular lysis being unsuccessful, these protein structures are taken within the cells where a greater concentration of enzymes may destroy them. *Fundamentally, serum bacteriolysis and phagocytosis seem to be the same—in each case specific antibody sensi-*

[41] Went, *Z. Immunitäts.*, 37:408 (1923), 39:76 (1924).
[42] Hektoen, *J. Infectious Diseases*, 6:78 (1909); 12:1 (1913).
[43] *Sperimentale*, 71:459 (1918).
[44] Walravens, *Bull. acad. roy. méd., belg.*, 30:204 (1920).
[45] *Brit. Med. J.*, 1923, Dec. 1, p. 1033.
[46] *Arch. Sci. Biol.*, 15:145 (1910); 16:161 (1911).

tization prepares the bacterium for lysis by enzymes, either inside or outside the cells that furnish the lytic enzyme.

As yet nothing is known concerning the change brought about in the bacteria by the opsonin, although it has been established that it is the bacteria that are modified and not the leucocytes. The chemical nature of the opsonins is likewise unknown, except that they may combine with certain inorganic ions and are then inert (Hektoen and Ruediger),[47] since addition of $CaCl_2$, $BaCl_2$, $SrCl_2$, $MgCl_2$, K_2SO_4, $NaHCO_3$, sodium oxalate and potassium ferrocyanide, inhibits the opsonic effect of serum. On the contrary, calcium salts stimulate the phagocytic effect of leucocytes, salts of barium and strontium being inactive.[48, 49] In common with other immune bodies, opsonins are thrown down in the soluble serum globulins.[50] Höber and Kanai[51] observed that serum globulin itself increases the phagocytosis of carbon particles, apparently by facilitating adsorption, and the favorable influence of calcium ions they attribute to its effects in increasing adsorption. They suggest that the increase in serum globulin observed in infectious diseases serves to facilitate phagocytosis, but the specificity of the increase in opsonins is apparently not taken into account by them, and does not harmonize with these simple physical explanations.

Opsonins are very sensitive to acids and alkalies and their maximum effect is at the neutral point.[52] However, treatment of either the bacteria or the leucocytes with very weak acids or alkalies, increases the rate and amount of phagocytosis.[53] It is said that opsonins may be developed by immunizing against substances practically free from protein, e.g., melanin granules.[54] Injection of nuclein preparations may increase the amount of opsonin present in the blood.[55] Cholesterol in excess diminishes phagocytosis, but apparently through its action on the leucocytes.[56] Both the sensitization of bacteria and their ingestion by leucocytes, either with or without sensitization, take place in accordance with the laws regulating an adsorption process, according to Ledingham,[57] but his observations have been disputed by Fenn.[58]

[47] *J. Infectious Diseases*, **2**:129 (1905).
[48] *Biochem. Z.*, **26**:66 (1910).
[49] Hamburger, *Biochem. Z.*, **24**:470 (1910).
[50] *See* Simon *et al.*, *J. Exp. Med.*, **8**:651 (1906); Heinemann and Gatewood, *J. Infectious Diseases*, **10**:416 (1912).
[51] *Arch. ges. Physiol.*, **198**:401 (1923).
[52] Noguchi, *J. Exp. Med.*, **9**:455 (1907).
[53] Oker-Blum, *Z. Immunitäts.*, **14**:485 (1912); Schütze, *J. Hyg.*, **14**:201 (1914).
[54] Ledingham, *Z. Immunitäts.*, **3**:119 (1909).
[55] Bedson, *J. Path. Bact.*, **19**:197 (1914).
[56] Dewey and Nuzum, *J. Infectious Diseases*, **15**:472 (1914).
[57] *J. Hyg.*, **12**:320 (1912).
[58] *J. Gen. Physiol.*, **3**:465 (1921).

Mudd [59] has shown that tubercle bacilli, when opsonized with immune serum, exhibit increased cohesion, a reduction in the surface potential difference, and a change in the bacterial surface whereby it becomes readily wet by water, whereas untreated tubercle bacilli are wet by oil. These changes are usually associated with a corresponding degree of increased phagocytosis, apparently depending on these surface changes, which presumably result from the deposition on the bacterial surface of the serum components which constitute the opsonizing antibody. This is quite similar to the observation that in agglutination of bacteria by immune serum the agglutinating antibody is deposited on the surface of the bacteria.

Recapitulation

In the actual defense of the body against infection, the cells of the blood and tissues play a part which is, in all probability, far more important than that played by the blood plasma and tissue fluids alone. The attraction to the point of infection of wandering cells, both the leucocytes of the blood and the unfixed tissue cells, is induced by the action of chemical substances produced by the infectious agent or by the injured tissue cells, but the chemical nature of these attracting chemotactic substances is not known. Their effect on the cells resembles much that of a substance lowering surface tension, and experiments in which oil droplets and other immiscible droplets suspended in fluid are acted upon by substances lowering surface tension, show that it is possible to cause such droplets to simulate in a most remarkable way the behavior of leucocytes and amebæ. However, it is not established that all the manifestations of chemotaxis and phagocytosis depend on surface tension alterations.

Phagocytosis, the ingestion of the bacteria or foreign particles by the cells, seems to be merely an extension of the process of chemotaxis, the cell flowing about the particle to which it has been attracted. In order that phagocytosis may take place it is necessary that electrolytes be present. The phagocytosis of bacteria is greatly augmented by the presence of specific substances which are increased in amount by immunization. These activating substances, called opsonins or tropins, seem to be true antibodies, but it has not yet been determined whether they are different from the antibodies responsible for the other immunological reactions. Neither has it been determined to how great an extent

[59] Mudd *et al.*, "Colloid Symposium Monograph," New York, The Chemical Catalog Co., Inc., 1928, p. 131.

the infecting bacteria are devitalized by the antibodies of the plasma before they are taken up by the phagocytes, and how much of the destruction of virulent bacteria is accomplished within the phagocytic cell.

Fundamentally, serum bacteriolysis and bacterial destruction by phagocytosis seem to be the same—in each case specific antibody sensitization prepares the bacterium for lysis by enzymes, either inside or outside the cells that furnish the lytic enzyme.

It would seem that opsonization and phagocytosis constitute but one of a series of similar processes by which foreign proteins are removed from the blood and tissue; i.e., by lysis by extracellular enzymes when this is possible, as it is with simple protein aggregates (albuminolysis) and with some of the more labile cells (hemolysis, bacteriolysis); but in the case of more resistant structures, notably Gram-positive cocci and acid-fast bacilli, extracellular lysis being unsuccessful, these protein structures are taken within the cells where a greater concentration of enzymes may destroy them.

CHAPTER XI

RESISTANCE TO NON-ANTIGENIC POISONS

Although it is generally recognized that the human organism acquires through habituation an increased tolerance to certain drugs and poisons of non-protein nature, this never reaches so marked a degree as to be properly characterized as immunity. The habitual alcoholic still exhibits the effects of his cups when his limit is passed, and the morphino-maniac obtains the same results as the unaccustomed subject if he receives enough morphine; the reputed maximum tolerance observed in arsenic eaters is at best but three to four times the minimum and less than the certainly fatal dose. Nevertheless some means of defense against certain non-antigenic poisons does exist beyond any doubt, although in no single case has the mechanism been established beyond dispute. That the immunity does not depend upon the formation of specific antibodies is certain, for the blood of the protected individual cannot protect another animal against the poison. To be sure, Landsteiner has shown that simple organic and inorganic radicals, including arsenicals, when combined with proteins so modify the antigenic capacity of these proteins that the antibodies engendered in animals immunized with them will specifically unite with these non-protein radicals and with compounds containing them,[1] which suggests a possible way in which immunity might be developed against non-antigenic poisons. Furthermore, it has been found that sometimes the blood serum of persons exhibiting hypersensitivity to mercury, bismuth, and salvarsan, is capable of sensitizing the skin of guinea pigs to these substances,[2] as if such hypersensitivity depended on anaphylactic antibodies (q.v.). Nevertheless, carefully controlled experiments have so far failed to demonstrate the existence of antibodies capable of neutralizing simple organic and inorganic chemical poisons.

Narcotic Poisons

A few observers have claimed that the serum of animals immunized to morphine will neutralize to some degree the toxic effects of mor-

[1] *J. Exp. Med.*, 39:631 (1924).
[2] Biberstein, *Z. Immunität.*, 48:297 (1926).

phine, but these results have not been generally substantiated.[3] Others have claimed that increased oxidative powers are developed under the stimulation of the poison, which permits of its more rapid destruction, especially in the liver, but the experimental support of this hypothesis is slight. Still another idea is that, at least in the case of morphine, decomposition products are produced, and accumulate in the body, which neutralize physiologically to some extent the morphine itself;[4] this hypothesis can scarcely be applied to arsenic and alcohol tolerance. It has been found that in animals habituated to morphine there is an increased power to destroy morphine,[5] but, nevertheless, the blood of such animals still contains quantities of morphine toxic for normal animals, so there must be a certain refractoriness or cellular immunity in addition (Rübsamen).

Schweisheimer [6] has shown that when chronic alcoholics and total abstainers are given equal quantities of alcohol, the alcohol content of the blood reaches a higher level, and persists for a longer time at a high level, in the abstainers.[7] Apparently the alcohol-habituated organism can destroy alcohol more readily, presumably through more rapid oxidation.[8] However, other factors are involved in alcohol tolerance, for with equal quantities of alcohol in the blood the abstainers show a more marked intoxication than the habitual drinker, and the confirmed alcoholic also shows a relative insusceptibility to a wide range of other narcotics, e.g., ether, chloroform, chloral, veronal. So, too, in morphine tolerance any general resistance through augmented oxidation seems inadequate in view of the specific increase in the tolerance of the respiratory center observed in this condition.[9] Also it has been sometimes found that tolerance to one drug may be accompanied by tolerance to other drugs exerting similar physiological action,[10] although even this is not altogether accepted.[11] Tolerance may be of transient character, as observed in the clinical use of nitrites [11a] and the immunity of miners to "dynamite headache" only while frequently exposed.

Du Mez [12] has reviewed the literature on morphine tolerance, and

[3] Pellini and Greenfield, *Arch. Int. Med.*, **26**:279 (1920); **33**:547 (1924).
[4] A. Valenti, *Arch. exp. Path. Pharm.*, **75**:437 (1914). Not corroborated by Pellini and Greenfield.[3]
[5] Takayanagi. *Arch. exp. Path. Pharm.*, **102**:176 (1924).
[6] *Deut. Arch. klin. Med.*, **109**:271 (1913).
[7] Albino rats having ten generations of alcoholic ancestry showed no increased resistance to alcohol [Hanson and Hays, *Am. Naturalist*, **61**:43 (1927)].
[8] *See also* Völtz and Dietrich, *Biochem. Z.*, **68**:118 (1915). J. Hirsch, *ibid.*, **77**:129 (1916).
[9] Van Dongen, *Arch. ges. Physiol.*, **162**:54 (1915).
[10] Myers, *J. Pharmacol. Exp. Therap.*, **8**:417 (1916).
[11] Biberfeld, *Biochem. Z.*, **77**:283 (1916).
[11a] Stewart, *J. Am. Med. Assoc.*, **44**:1678 (1905).
[12] *J. Am. Med. Assoc.*, **72**:1069 (1919); see also DuMez and Kolb, *U. S. Pub. Health Service Reports*, **40**:548 (1925); Scott *et al.*, *Arch. Int. Med.*, **35**:472 (1925).

summarizes the evidence as follows: "The only knowledge of a positive nature that we have at present concerning these problems is that the different organs and centers of the body acquire tolerance to morphine and heroin to a different degree and with varied degrees of readiness; that these drugs as such are excreted in the feces in diminishing amounts during the period of acquiring tolerance; and that there is evidently present in the blood serum of tolerant animals (dogs) during periods of abstinence a substance or substances which, when injected into normal animals of the same species, causes the appearance of symptoms identical with the so-called withdrawal phenomena. Whether or not the disappearance of these drugs from the feces is due to their increased destruction in the organism is still an unsettled question. It has not been proved that the destruction of morphine in the organism, if it does take place to an increased degree, is a causative factor in the production of tolerance. It may be only a concomitant phenomenon."

A consideration of all the evidence on acquired tolerance to hypnotics led Gunn [13] to the conclusion that at present it is permissible to make the following deductions:

"(1) Among drugs generally, it is chiefly to depressants of the central nervous system that tolerance can be acquired,—to alcohol, chloral, morphine, etc., rather than to, e.g., codeine, strychnine or other alkaloids. (2) When a substance combines a depressant action on certain parts of the nervous system with a stimulant action on other parts of the nervous system or with other physiological actions, it is to the former chiefly or only that tolerance is established.[14] The experiments of Van Egmond, Van Dongen and Tamura with morphine, of Langer with heroin, and of Biberfeld with paracodeine, are all in agreement on this point. (3) In regard to these hypnotics, therefore, acquired tolerance is largely if not entirely due to the fact that certain parts of the central nervous system can acquire an increased resistance to the action of depressants. (4) It may not be accidental to this that tolerance can be acquired the more easily in different animals in proportion to the higher development of the brain, e.g., in the order man, dog, rabbit, frog. (5) The fact that some degree of tolerance can be acquired to alcohol, chloral, veronal, morphine, cannabisindica, hyoscine, substances with little or no chemical similarity but which agree in being cerebral depressants, suggests strongly that tolerance

[13] *Physiol. Rev.*, 3:41 (1923).

[14] The stimulating effect may even be increased [Jöel and Ettinger, *Arch. exp. Path. Pharm.*, 115:334 (1926)].

is more intimately connected with their actions on the nervous system, in which they agree, than with increased destruction of them, in the mechanism of which they must necessarily disagree."

Gunn is very sceptical as to the possibility that an animal can either acquire by habituation a destructive action which it did not originally possess, or increase a pre-existent destructive power. He calls attention to the fact that the condition of alkaptonuria, especially, shows that when there is a congenital absence of the enzyme which destroys homogentisic acid, this ferment action is never gained throughout life, though the substance to be destroyed is being constantly formed from the food. This seems to be one certain example of the complete inability of the body to form a new enzyme in response to habituation.

In regard to the second question, he says: "It is quite possible that ferment activity can be artificially increased, because animals fed for a long period on one particular kind of food may develop an abnormal ferment activity for this particular food. The difficulty in regard to, e.g., alkaloids, would seem to be this: The ferment that destroys them is one that presumably normally destroys some other substance and it is difficult to imagine how this ferment can be increased by the minute quantities of alkaloid (e.g., of nicotine) which enter the circulation. In other words, one would imagine that there would be even larger fluctuations from day to day in the amount of substance that the ferment normally destroys than would be afforded by the addition of the alkaloid. At all events these considerations would seem to urge a caution against accepting results which have claimed to show an augmentation of destruction of alkaloids given in small amounts and for short periods and especially those results which claim that a new power of destruction can be originated by habituation."

Arsenic Habituation

The increase in tolerance observed in arsenic eaters is so low that its existence has been disputed by some, although most writers on the subject accept the existence of greater or less tolerance. Cloetta [15] especially has investigated this subject and attributes the resistance entirely to decreased absorption, for he found that the longer arsenic is taken the less appears in the urine and the more is excreted in the feces. There is no tissue immunity, for habituation to arsenic by mouth, according to Cloetta, does not increase resistance to subcutaneous injections, nor does repeated injection of sublethal doses lead to any tolerance. He

[15] *Arch. exp. Path. Pharm.*, **54**:196 (1906); *Correspondbl. Schweizer Aerzte*, **41**:737 (1911).

could obtain no tolerance to antimony in experimental animals.[16] Joachimoglu [17] could not corroborate Cloetta's finding of a decreased total absorption in habituated animals, but he did find that they suffered less damage to the epithelium of the digestive tract than normal animals, in which absorption is therefore more rapid even if not in greater total amount.

Schwartze [18] has reviewed critically the literature on experimental habituation to arsenic, and calls attention to the possibility that the supposed positive results, such as those of Cloetta, may depend on the marked insolubility of the arsenious oxide used in such experiments, for a large proportion of this substance given by mouth may remain unabsorbed and be eliminated in the feces, much depending on the size of the particles of the poison. No successful habituation seems to have been obtained with more soluble arsenical compounds.[19] These valid criticisms justify Schwartze's statement that habituation of higher animals to arsenic has not been proved, although this failure does not prove that it cannot exist. Certainly the experiments of Wilson [20] on tissue cultures indicate that tissue cells actually may acquire an increased tolerance to copper sulfate and sodium arsenite when grown in weak solutions of these poisons, just as had been previously found for unicellular organisms grown in alcohol and corrosive sublimate. In these tissue cultures there can be no question of serum antibody protection; the resistance must depend on changes in the cells themselves.[21]

Also, it is an interesting fact that many pathogenic organisms acquire a more or less specific immunity to both organic and inorganic parasiticidal drugs, e.g., the development of strains of trypanosomes immune specifically to arsenicals and to dyes, and of bacteria resistant to antiseptics.[22]

Defensive Mechanisms

It is possible, of course, that the cell constituents with which the poisons ordinarily combine are produced in increased amounts under the stimulus of the poison, just as they are supposed to be in the case of immunization with toxins, with the difference that the combining substances are not thrown off into the blood. For example, it has been

[16] *Arch. exp. Path. Pharm.*, 64:352 (1911).
[17] *Arch. exp. Path. Pharm.*, 79:419 (1916).
[18] *J. Pharm. Exp. Therap.*, 20:181 (1922).
[19] *See also* Schwartze and Munch, *J. Pharm. Exp. Therap.*, 28:351 (1926); Issekutz and v. Végh, *Arch. exp. Path. Pharmakol.*, 114:206 (1926).
[20] *Bull. Johns Hopkins Hosp.*, 33:375 (1922).
[21] There is evidence of more or less tolerance or increased resistance being acquired to lead and other metallic poisons. [*See* Weller, *Arch. Int. Med.*, 39:45 (1927).]
[22] *See* Schnabel, *Klin. Wochschr.*, 3:566 (1924); Dale, *Physiol., Rev.*, 3:366 (1923).

claimed that arsenic is ordinarily combined and held in the liver by a nucleoprotein, and the suggestion has been made that in arsenic habitués this nucleoprotein is increased in amount, but this has not been proved. Again, the hemolytic poison, saponin, seems to act upon the cholesterol of the red corpuscles, and Kobert observed increased resistance to the action of saponin exhibited by the serum of immunized animals, which he attributed to an increased amount of cholesterol, perhaps liberated by the corpuscles decomposed by the injected poison, or perhaps given out to the blood in excess by the tissues. Wohlgemuth [23] has also suggested that in the case of poisoning with large amounts of substances which combine with glycuronic acid (e.g., lysol), excessive quantities of this substance are formed by the cells and excreted into the blood, where they neutralize the poisons in much the same manner as the antitoxins neutralize toxins. Sherwin believes that under necessity the animal body may build up out of waste nitrogenous products (urea or its antecedents) the necessary amino acid for detoxication of certain aromatic fatty acids.[24] His results indicate that glycine and glutamine may thus be built up from urea by mammalian organisms, while fowls form ornithine for the same purpose. Nevertheless, if such adaptations do exist they are probably of no great range or persistence, for it is apparent that the amount of combination of simple chemical poisons which may be thus detoxicated is very limited, and that it is usually easily possible to introduce an excess which goes uncombined.

There are, to be sure, many chemical means of defense against non-antigenic poisons, e.g., the precipitation of inorganic poisons by H_2S in the alimentary canal; or the accumulation of poisons in tissues where they do relatively little harm, e.g., the fixation of arsenic in the liver. But these means are not ordinarily augmented by repeated introduction of the poisons. These non-specific chemical defensive mechanisms I have discussed more in detail elsewhere,[25] and the fate of foreign organic compounds in the animal body has been thoroughly reviewed by Sherwin.[26] The general facts may be summarized as follows:

Against non-antigenic poisons the variety of reactions and the variety of defensive substances are both remarkably small in number. The reactions are: Oxidation and reduction, hydration and dehydration.

[23] *Biochem. Z.*, 1:161 (1906).
[24] Shiple and Sherwin, *J. Am. Chem. Soc.*, 44:618 (1922); Power and Sherwin, *Arch. Int Med.*, 39:60 (1927).
[25] Wells, "Chemical Pathology," 5th Edition 1925, Chapter XII. W. B. Saunders Co., Philadelphia.
[26] *Physiol. Rev.*, 2:238 (1922)

and perhaps simple addition (methylation).[27] The chief known protective substances are the alkalies of the blood, proteins, hydrogen sulfide, sulfuric acid, glycine, glutamine, urea, cysteine, bile acids, glycuronic acid, and acetic acid.[28] All these substances are normally present in the body, and none of them is specific against any one poison, but each combines with several poisons. According to Sherwin, different species of animals may employ an entirely different reaction for the detoxication of a certain chemical substance; e.g., phenyl acetic acid in man is combined with glycine, but in birds with ornithine.

As far as we know, no particular increase in the neutralizing substances results from the administration of inorganic or organic poisons. The body does not appear ordinarily to produce any excessive amounts of sulfuric acid in phenol poisoning, or of glycine when benzoic acid is administered. Both of these neutralizing substances or their antecedents are present in the body normally, and as much as is available combines with the poison; if there is not enough, the remaining poison combines with something else or goes uncombined. In other words, the neutralizing substances described above do not appear to be the result of any special adaptation to meet a pathological condition. They are present in the body as a result of normal metabolism; they have an affinity for various chemical substances, some of which happen to be poisons; if these poisons happen to enter the body, they may be combined and neutralized to some extent, but, as a rule, very incompletely. There appears to be no elaborate and persisting process of defense against the chemically simple poisons, such as seems to be called into action by bacterial infection, and hence a degree of resistance or immunity similar to that present after an attack of scarlet fever or smallpox does not exist for strychnine or phosphorus.

There exists also a natural tolerance to many poisons, which may differ strikingly in different species, and is therefore of hereditary origin, just as some types of hypersensitivity are hereditary. Gunn [10] has reviewed the literature of this subject and calls attention to the fact that with a great many drugs, e.g., most heavy metals, quinine, chloral, phenol, etc., the minimum lethal dose per kilogram is very nearly the same for all species of warm-blooded animals; on the other hand, with some drugs the difference may be as much as 100 to 1 or

[27] Alterations in the effect of substances on surface tension by chemical change may possibly be responsible for reduced toxicity [Berczeller, *Biochem. Z.*, 84:75 (1917); see critique by Rose and Sherwin, *J. Biol. Chem.*, 68:565 (1926)].

[28] In many cases these substances, most of which are not soluble in lipoids, detoxicate lipoid-soluble substances, possibly through reducing their solubility and cell permeability [Schüller, *Arch. exp. Path. Pharmakol.*, 106:265 (1925)].

more. When one species shows a high resistance as compared with the majority, it is regarded as congenital tolerance in the former; if a low resistance, congenital intolerance or hypersusceptibility. Among the examples of congenital tolerance may be mentioned that of the toad to the digitalis-like poison of the toad's dermal glands as well as to digitalis itself and other poisons of the digitalis group, also the resistance of rats and grass snakes to these same poisons. This tolerance depends upon a specific lack of susceptibility of the heart muscle of these animals, not accompanied by increased tolerance to many other poisons. Even more striking in degree is the resistance of the hedgehog to cantharidin, for Ellinger [29] calculated that one gram of cantharidin is a fatal dose for 20,000 kgm. of man, 500 kgm. of rabbit, and 7 kgm. of hedgehog. He found that this tolerance was not due to defective absorption, for it was true also of intravenous injection. It was not due to chemical change or neutralization in the body because cantharidin could be recovered unaltered in the urine. His experiments showed clearly that the tolerance was due, partly at least, to an insusceptibility of the kidney cells to the action of cantharidin. Also to be mentioned in this list is the congenital tolerance of the rabbit and frog to atropine, which seems to depend on a particular capacity of the liver or of the serum [30] of these animals to destroy this alkaloid.

Recapitulation

The recognized resistance that may be acquired to simple chemical poisons, such as morphine, alcohol, nicotine, and possibly arsenic, is essentially different from the immunity developed against antigenic poisons. The degree of resistance is relatively low, is not altogether specific, does not persist for long, and is not accompanied by or dependent upon the presence in the blood of specific antibodies which neutralize or unite with the poison. To some degree organic poisons may be overcome by an augmented power to oxidize them, but in part the resistance depends upon an increased tolerance of the tissues for these poisons. Among the drugs, it is chiefly against those with a depressant action on the central nervous tissues that tolerance is acquired, stimulants not leading to habituation. The resistance acquired to arsenic seems to be much less than is generally believed, and perhaps does not exist in the higher animals, but since unicellular forms and mammalian cells in tissue cultures may develop tolerance for arsenic, it seems probable that the mammalian organism may develop some resistance.

[29] *Arch. exp. Path. Pharmakol.*, 45:89 (1900).
[30] La Barre, *J. Pharm. Exp. Therap.*, 26:259 (1925).

For the most part such protection as there is against simple, non-antigenic poisons is accidental and not developed specifically against the poison. It depends on the presence in the body of substances which happen to combine chemically with the poison, e.g., precipitation of minerals by H_2S in the intestine, or union of phenol with sulfates in the blood and tissues. The protection afforded by such reactions is limited to the amount of available material, and no adaptation to meet a new situation is involved and no enhanced resistance follows single or repeated sublethal poisoning. The natural, inherited immunity exhibited by some animals against chemicals poisonous for other species, although often very marked, has not been explained.

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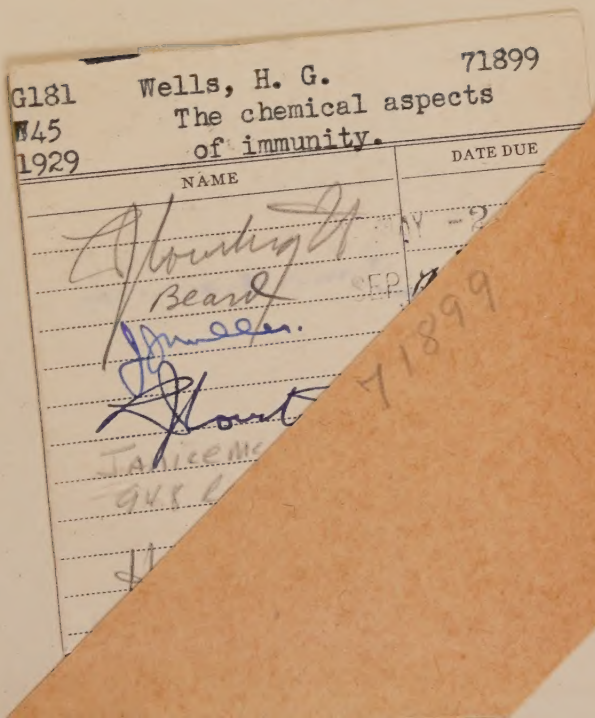
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